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# *The Journal of Laboratory and Clinical Medicine*

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## *CLINICAL AND EXPERIMENTAL*

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### A CLINICAL METHOD FOR STUDYING THE FACTOR OF HUMAN RELATIONS IN DISEASE\*

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THE technique of measuring human relations which we are to describe evolved as a result of the need for a method of objectively describing and analyzing data obtained in anthropologic field studies and interviews. It has been described in detail elsewhere.<sup>1,2</sup> It provides the sociologist, psychologist, and anthropologist with a useful means of visualizing at least some of the dynamics involved in interpersonal relations. The physician as well will find this method of some value in understanding those baffling problems in interpersonal relations which invariably appear in the practice of medicine.

Often the doctor gives advice to his patients which is tempered by a consideration of each patient's personality. Even before the development of psychiatry the physician considered this factor. The family doctor realized, by virtue of his long-time association with families, that the manner in which the members of a family group adjusted themselves to each other was an important factor in precipitating emotional upsets, which might not only cause disease but were fundamental in determining the future course of illness and the patient's manner of recovery. With the development of his "life chart" technique for presenting case histories, Adolf Meyer<sup>3</sup> early emphasized the basic interrelation of environment and mental disease. Today interpersonal relations are recognized as being a most important part of environment, and as diagnosis and treatment of disease have progressed, this factor has gradually been unmasked so that it is now clinically recognized as being a causal or precipitating factor in many illnesses and a factor modifying the progress of all disease.

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We offer here a method which enables the physician to understand more clearly the problems of interpersonal relations which his patients present. By its use the concept of interpersonal relations becomes more tangible, becomes subject to measurement. Psychosomatic medicine is a new and broad field in which the wisdom and skill of the clinician, psychiatrist, social worker, and others are taxed to the utmost in attempts to bring relief to the patient. The psychoneuroses likewise demand special skills. The clinical measurement of *interaction* by means of recording the time relations in interviews of the doctor with the patient and with others in the patient's environment will aid in the understanding and treatment of these confusing psychosomatic and psychoneurotic disorders.

#### THEORETICAL CONSIDERATIONS

Interaction is defined as that portion of a person's total activity which is immediately followed or preceded by activity on the part of another person. A conversation between the doctor and the patient represents a sample of the daily interaction of each. Almost all of an individual's life is spent in interaction with other individuals in institutions, such as the home, business, clubs, et cetera. The most striking feature of the interaction of individuals is that each individual tends to maintain an equilibrium with respect to the quantity and type of interaction in which he partakes. Human beings have the property of maintaining a fairly constant amount of interaction in their interpersonal relations and of compensating for any changes in these relations by appropriate reactions. This concept of equilibrium is most useful when considering psychiatric problems in general practice.

The internal environment of each individual is maintained in a state of homeostasis by the action of the autonomic nervous system and, as Cannon and his associates<sup>5</sup> have convincingly shown, this constancy of the internal environment is maintained against the disturbing effect of changes in the external environment. Disturbances of the equilibrium which exists in interpersonal relations result in an activation of the autonomic nervous system. Emotion is an outward expression of this activation. The mechanisms which a person possesses to maintain himself in a state of homeostasis despite changes in his external environment are no less active during times when great stress is being brought against him by other persons than when he finds himself, for instance, inadequately protected from cold. Many of the symptoms of the psychosomatic and psychoneurotic disorders arise from this activated autonomic nervous system. The nature of the responses to stimuli from other persons is determined not only by conditioning or "past experience" and from the resultant cortical activity, but also by the autonomic nervous system which "endeavors" to maintain each person in his characteristic equilibrium with other people and the rest of the external environment.

Not only may each individual be regarded as being in a state of equilibrium in his interpersonal relations, but each of these equilibrium states will differ from individual to individual. Some persons may require a great amount of activity with others, while some may be content with a rather small amount of daily activity. Some persons may need more originaive activity;

these represent the "extravert" type of person. This equilibrium is a relatively stable property of each individual. It is developed in childhood within the family institution, and its form depends upon the relationship of the child to the various members of the family. It undergoes modification during the school years, adolescence, and young adulthood as the individual broadens his field of interpersonal relations. The stability of an equilibrium is reckoned by the degree to which emotional reactions *fail* to be called forth during interpersonal relations. Great stresses upon an equilibrium, such as marriage, death, birth, loss of job, et cetera, will cause profound emotional reactions. However, a stable equilibrium in normal day-to-day living will find little strong emotional coloring to interpersonal relations. "Emotional instability" may be considered as merely the reflection of an unstable equilibrium. The development of an unstable equilibrium is a most complex phenomenon, as yet far from understood, and involving heredity, conditioning, sudden environmental changes, and so forth—in fact, involving a consideration of the individual's present situation, his past, his whole life experience. It is this fact that Adolf Meyer emphasized forty years ago.

Laboratory evidence of the validity of these concepts is derived from several sources. Spontaneous activity has been described by Richter<sup>6</sup> as that activity which animals exhibit when environmental stimuli are removed. He has demonstrated that rhythmic variations in amount of spontaneous activity exists in rats in relation to hunger, thirst, elimination, and the estrous cycle. Stier<sup>7</sup> has demonstrated similar rhythms and has shown that the basic amounts of daily activity in newborn thermolabile mice varies with changes in temperature. This work corresponds to that done by Crozier<sup>4</sup> and others, who have demonstrated, with changes in temperature, changes in the rates of various specialized activities in poikilothermic animals such as chirping of crickets and breathing movements of fish. The relations of activity rate and temperature have been equated and shown to have properties indicating control by relatively simple chemical reactions. Hoagland<sup>8</sup> has termed such reactions "pacemaker reactions," and postulates the presence of a system of such reactions involving inorganic catalysts as controlling the various activity rates of organisms.

In the higher organisms the endocrine and homeostatic-controlling mechanisms naturally serve to mask the presence of such basic patterns. Changes in activity resulting from changes in the external environment result in marked deviations from any basic activity rate. Nevertheless it is of some interest to postulate the presence of this basic rate. Richter has demonstrated species differences in amounts of daily spontaneous activity, but has not to our knowledge shown that there is a racial difference. However, evidence from other sources indicates that basic interaction rates are inherited. One of us (W. T. V., Jr.)<sup>9</sup> has found that highly inbred white mice differ markedly in interaction rates from inbred brown mice and also that the white mice are better equipped to withstand diminution of oxygen tension than are the brown mice. Scott<sup>10</sup> has observed differences in the mating behavior of various strains of white and brown mice; this also suggests hereditary differences in interaction.

Individual variations in this basic rate within homogenous strains are probably the result of, first, variations in the external environment, and, second, past experience of conditioning, and, third, developmental changes involving

primarily the endocrine system. Temporary changes in interaction rate which are usually compensated for by the individual and which involve emotional reactions are a result of the first of these three, and changes in equilibrium which occur over a number of years as the individual grows and is exposed to new situations involve the development of interaction patterns and rates due to the factor of conditioning. Allee<sup>11</sup> has studied many mice of different races and has, by virtue of the racial differences in behavior reported by Scott, been able to carry out controlled experiments demonstrating that variations in conditioning dose affect activity rates. He has placed this study on a quantitative basis, and has shown that to develop, for instance, a high activity rate with initiative characteristics, it takes a lesser amount of a certain conditioning stimulus in the more dominant race of brown mice than it does with the more submissive white mice. Activity changes paralleling changes in endocrine balance have been noted by Richter in the laboratory and are seen clinically during the menopause, adolescence, and most strikingly during testosterone therapy for eunuchoidism<sup>12</sup> and other disorders.

All the evidence points to the presence of these four main factors affecting activity and interaction: heredity, development or endocrine balance, environment, and conditioning. It is impossible to consider one without the other. The effects of all four blend, resulting in the most complex integration of stimuli and responses, the behavior of the living being.

#### THE INTERACTION CHRONOGRAPH AND MEASUREMENT OF INTERACTION

With this understanding of interaction and of the equilibrium present in interpersonal relations, we can proceed to describe the method for measuring interaction and for visualizing the equilibrium which a patient may exhibit. In an interview with a physician, of course, the patient may not be exhibiting the equilibrium which is quite characteristic of his home or business interaction. This must be determined by a carefully taken history, such as has recently been described by Saslow and Chapple.<sup>13</sup> However, since the relation of the physician to others is of a rather constant nature from the point of view of interaction, it is possible to compare the interaction of various patients with the doctor and derive valuable data therefrom.

Fig. 1 illustrates the interaction of one person with another during an ordinary conversation. Interaction, as we have said above, is that part of a person's activity which is preceded or followed by activity on the part of another individual. In any conversation, interaction consists of alternating talking and silence. Ordinarily, when one person talks, the other is silent. However, at times one person may interrupt the other, and both will talk at the same time until one of them stops; or one person may fail to respond when the other stops talking, and both will be silent till one or the other begins to speak again. In a conversation, then, we can measure the consecutive lengths of time each person talks and is silent, the length of time he interrupts the other or fails to respond, and the frequency with which each outtalks the other or starts acting after a double silence. If neither person interrupts the other or fails to respond, we can regard them as well adjusted, since each is able to synchronize his interaction pattern with the other within short inter-

vals of time. The more interruptions and failures to respond and the longer they last, the poorer is the adjustment. If these durations of actions and silences are plotted in such a way that action is marked by an increase and the following silence by a decrease, we can then obtain a graphical representation of the interaction. In Fig. 1 along the ordinate is measured the excess in duration of actions of the subject over duration of silences, expressed in seconds. Along the abscissa are plotted the interaction units, that is, the intervals which include one action and its successive silence. As a person becomes more talkative relative to his silences, the activity curve ( $A$  minus  $S$ ) moves up; as he is silent, it moves down. It can be seen in Fig. 1 that the individual was very talkative.

The four curves shown in the graph represent the different measures taken during the interaction of one individual with another. They are in the form of an alternating series, namely, a series in which the actions are reckoned as positive and the silences negative, and the series of actions and silences is summed cumulatively. The use of the alternating series in describing interaction is based upon our basic assumption that the amount of activity and inactivity of an individual bears a constant relationship, that an equilibrium exists. If there is an excess of activity at one time during the day, there will be an increase in inactivity later in the day, maintaining a rather constant daily rate of activity. These variations in output of activity have been described by Stier, Richter, and Skinner.<sup>6, 7, 14</sup> Since the interaction situation requires an alternation between the action of one person and the action of another, some kind of balance has to be obtained in the durations of activity for each individual. The alternating series provides a continuous measure of the changes in output from individual to individual and of the adjustments which each has to make to the other.

The first curve, marked in a solid black line, is the *activity curve* of the individual ( $A$  minus  $S$ ), the duration of his actions minus duration of his silences summed in alternating series. In the same manner, the second curve represents his adjustment to the other person and is conveniently called the patient's *adjustment curve* ( $U$  minus  $W$ ). It consists of the duration of the patient's interruptions of the doctor minus the duration of his failures to respond. The third curve ( $V$  minus  $X$ ) is the corresponding *adjustment curve* for the doctor, the duration of his interruptions minus the duration of his failures to respond. The fourth curve is called the patient's *initiative curve* (bar minus no bar) and represents a count of the frequency with which the patient, after interrupting, outtalks the doctor or, after a period when both are silent, initiates the activity minus those instances when the doctor outtalks the patient or initiates action after a silence. It measures the frequency or degree of dominance of one individual or the other.

The apparatus used to measure interaction is called the Interaction Chronograph. It actually draws the four curves while the interview is in progress, so that as soon as the interview is over, the graph is immediately available. The machine is operated by a technician who has before her two keys, one for each of the persons interacting. When the patient talks, she presses his key and holds it down until he stops, when she releases it. When the doctor talks, she similarly operates his key. Through an electrical system, the appropriate

writing arm is activated and the curves rise and fall as the interview proceeds. In the illustrations the curves have been smoothed to simplify their appearance.

In Fig. 1 we see that the activity rate of the subject is relatively high, as noted by the positive slope of the A minus S curve. From these data alone,

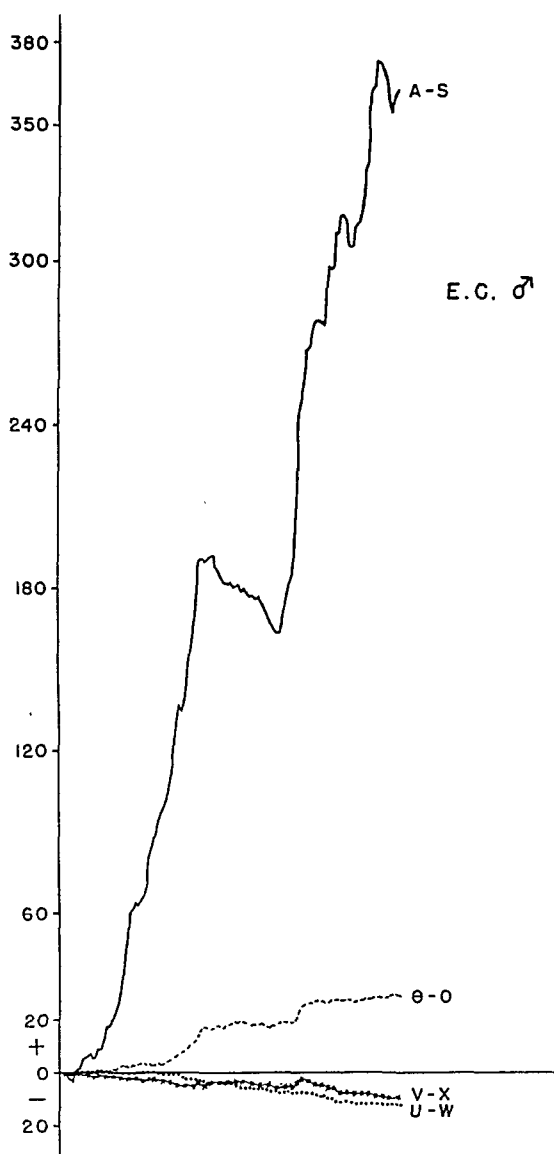


Fig. 1.

of course, it is impossible to tell whether or not this subject can maintain his high activity rate throughout the day. In order to maintain such an equilibrium he would need to interact with people who did not possess equally high rates; otherwise, we would find either that he would be continually interrupting his associates or that he or they would have to modify their natural rates very seriously in order to maintain an adjustment during conversations. In

this instance we see that satisfactory adjustment was maintained, as evidenced by the almost horizontal adjustment curves. The partner's adjustment curve ( $V$  minus  $X$ ) shows a slight downward trend. This means that he failed to respond more than he interrupted. The  $U$  minus  $W$  curve likewise has a slightly negative slope. Despite the high activity rate of one individual, there was very good adjustment during this conversation. At one point, it will be noted, the activity curve ( $A$  minus  $S$ ) descends; this means that the partner was talking an excessive amount during that period of time. If he had been quiet, this would be revealed by a concurrent fall in his adjustment curve ( $V$  minus  $X$ ). This naturally upset the equilibrium of the first individual, who, as we see, is at this time a very talkative person. His rapid activity rate was regained soon afterwards, however, as the activity curve resumes its previous degree of rise. It is to be noted that the slope of the initiative curve ( $\theta$  minus  $O$ ) increases at this same time; this means that though he was interrupted, the first person regained his dominance and exceeded his partner in talking. Here then is an instance where an individual in a state of equilibrium suffers a slight disturbance and compensates for the disturbance by outtalking the other person and forcing him to modify his rate, thus ending the disturbance and restoring his equilibrium.

#### MEDICAL APPLICATION OF THE CONCEPTS OF EQUILIBRIUM AND INTERACTION

Even in the simplest instances of illness, the physician will find it of utmost value to prescribe the type of environment most suited for an individual patient's equilibrium. This will involve a consideration of the patient's normal rate of activity, his capacity for adjusting to others, and his need to dominate or initiate the action. Thus, in Fig. 1, if it be considered a picture of that person's daily activity instead of simply thirty minutes of it, not only would he need to maintain a high activity rate but he would also have to dominate the interaction, initiate action whenever silences occur. If such a person is placed in an environment in which he has to respond to many actions and is not given an opportunity to initiate action to others, his equilibrium will be disturbed and an emotional reaction with altered behavior will probably be manifested. Situations such as this may be created when a patient's environment is changed due to illness. Patients ill at home are commonly surrounded by members of their family group with whom in normal times they would interact only for a few hours during the day. Unless the family is a very well-adjusted one, the patient may be thrown out of equilibrium because he now has to interact with the family group throughout the day. Interpersonal relations become an important consideration in the choice of a nurse or companion for the ill or convalescent patient; in such instances the patient is especially affected by any disturbance in his interpersonal relations, for he can no longer regain equilibrium through the channels of interaction normally open to him. These facts are of especial importance in the handling of patients who are characterized by extreme deviations in their interaction patterns, such as are most commonly seen in the psychosomatic and psychoneurotic disorders. In such cases the doctor should note carefully the characteristics of the interpersonal relations of the patient and those persons in his environment, for the degree to which the patient can be brought to cooperate and can be

made to feel emotionally stable in his interpersonal relations determines to a great extent the efficacy of treatment.

In those cases in which there is a great emotional factor, and indeed in all illnesses, the interpersonal relations of the physician himself with the patient bears scrutiny. All patients have different rates of interaction and different requirements for equilibrium, and yet each physician has clear-cut needs himself in terms of his relations to others. The physician must adapt himself to all types of persons and suffer upsets of his own equilibrium in order to maintain that of his patients in their interpersonal relations. He must understand the character of each patient's equilibrium and guard it against disturbance during the change in interpersonal environment necessitated by disease.

#### DISORDERED EQUILIBRIA

Great stresses in the interpersonal relations of an individual which result in temporary disequilibrium can usually be compensated for by the individual and his equilibrium restored. If these stresses continue, however, or if the compensatory reaction does not restore the equilibrium, the individual may gradually achieve a new equilibrium which is based upon an altered system of interpersonal relations. The most important stresses which bring about these profound modifications of the individual's personality are the major crises of life: death, birth, marriage, or entrance or departure from an institutionalized system of relations in which the individual has made an adjustment, such as entrance to school, or graduation, loss of a job, moving into a new community, and so forth. Each of these changes involves a change in the interaction of the individual, an increase, a decrease, a greater or less adjustment; and the reaction of the individual to these crises may bring about far-reaching effects in the kind of equilibrium he can attain. For these changes at the same time necessitate changes in the emotional adjustment of the individual through the operation of the autonomic nervous system, and the emotional changes may remain in varying degrees until a new equilibrium has been established.

Sometimes the new equilibrium is not a normal one. We may speak of it as a disordered equilibrium. The individual is unable to adjust himself to his new environment; the emotional disturbance, at first a normal physiologic phenomenon, fails to terminate, and over a period of months or years an equilibrium is attained which may involve a distortion of the interaction patterns and a malfunctioning of the autonomic nervous system. These disordered equilibria can develop not only after a major crisis, but also as a result of a cumulative series of minor crises during the early formative years of an individual's life. Some of these persons present themselves to the physician with symptoms referable to some function of the autonomic nervous system. Others consult the physician because their relation to the environment is not satisfactory, and still others are committed to medical care because their presence in society only serves to upset the equilibria of other people. Such disorders have been divided into three great groups, depending upon the predominating symptomatology, the psychotic, the psychosomatic, and the psychoneurotic disorders. Let us consider them now in the light of our understanding of interpersonal relations.

The psychosomatic disorders include many diseases in which "emotional disturbances" or "psychic traumas" play an important role. Certain cases of peptic ulcer, mucous colitis, ulcerative colitis, cardiospasm, and neurocirculatory asthenia fall in this group. Diseases and symptoms which often have a psychogenic factor include diarrhea, constipation, headache, fatigue, asthma, hay fever, neurodermite, Graves' disease, rheumatoid arthritis, and perhaps "the common cold" and essential hypertension. Herrmann<sup>15</sup> has said, "Disturbances of psychogenic and neurogenic origin make up at least one-third of all the disorders in cardiovascular consultation work." Internists in other fields are similarly faced with this problem of dealing with the emotional factors in disease. *Emphasis in this fact is found in a recent review of the psychosomatic disorders by Finesinger.*<sup>16</sup>

White, Cobb, and Jones<sup>17</sup> in their monograph on mucous colitis state that in no instance in their series of sixty cases was the patient the type of person who was able to carry on his daily activity *independent* of other people. The majority of patients exhibited characteristics of an "anxiety or tensional state." It seems as if these patients had developed a disordered equilibrium which made them peculiarly dependent upon a certain daily interaction rate, an equilibrium which stands only very slight stresses before severe disturbances of autonomic function result.

Vaughan<sup>18</sup> has written of psychogenic factors in the many allergic diseases. He says, "We may look upon the extrinsic allergic excitants such as pollens, foods, contact allergens, and physical agents as part of one's environment to which the normal person adequately adjusts himself with resulting immunity or freedom from symptoms." He further states, "... the clinical allergic response can nearly always be shown to be associated with the failure of satisfactory adjustment to environmental problems, even though the problem, or shall we say the allergenic excitant, be purely mental." His cases demonstrating psychogenic precipitating factors run the gamut of the common allergic manifestations, urticaria, vasomotor rhinitis, angioneurotic edema, asthma, and migraine.

Summarizing present knowledge of psychosomatic disorders, we see that many varied diseases which may be treated by the gastroenterologist, the cardiologist, the nose and throat man, the surgeon, or the allergist, have perhaps a common factor in the background, an inability of the patient to make a satisfactory adjustment to the external environment. The physician who is on the lookout for these environmental factors as etiologic agents in disease will quickly recognize their presence in various cases. Often the etiology is obscure, and a very thorough review of the life of the patient is necessary to reveal their presence. Often the physician may wish that he had some method by which he could appraise the "emotional state" of the patient, his relations with other people and how they affect him. The interaction chronogram represents such a tool by which a base line appraisal can be obtained. Supplemented with other data, such as the very carefully taken history of "emotional life," and social service reports, the physician has very definite information on which to evaluate the importance of interpersonal relations in etiology and treatment. Below are several case histories and interaction chronograms of patients with disordered equilibria and concurrent disease. They suggest



the usefulness of the interaction chronogram in treating these psychosomatic and psychoneurotic disorders.

THE INTERACTION CHRONOGRAM IN DIAGNOSIS AND TREATMENT

Fig. 2 illustrates a case in which the presence of environmental and emotional factors as dominant etiologic agents was quickly recognized. In con-

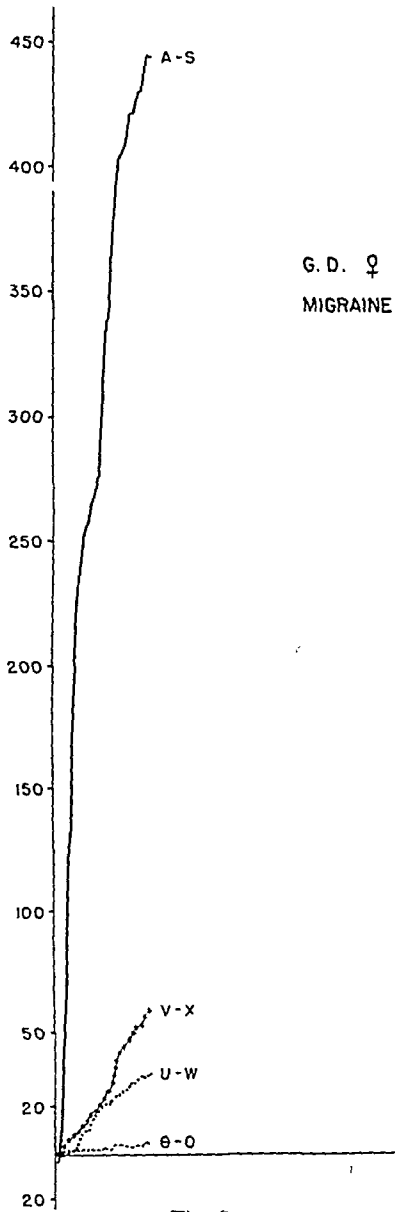


Fig. 2.

trast to Fig. 1, here we see a hyperactive person, with whom the psychiatrist was unable to maintain good adjustment. The patient's adjustment curve (U minus W) rises, meaning that she was interrupting the doctor. In turn,

his adjustment curve rises as he interrupts her. Finally, as the patient's activity curve maintains a constant high slope, the initiative curve also rises, indicating that the patient was ever dominant during the interview. Such a picture as this is decidedly abnormal and is, along with other types of disordered equilibria, typical of the activity of these people throughout the day

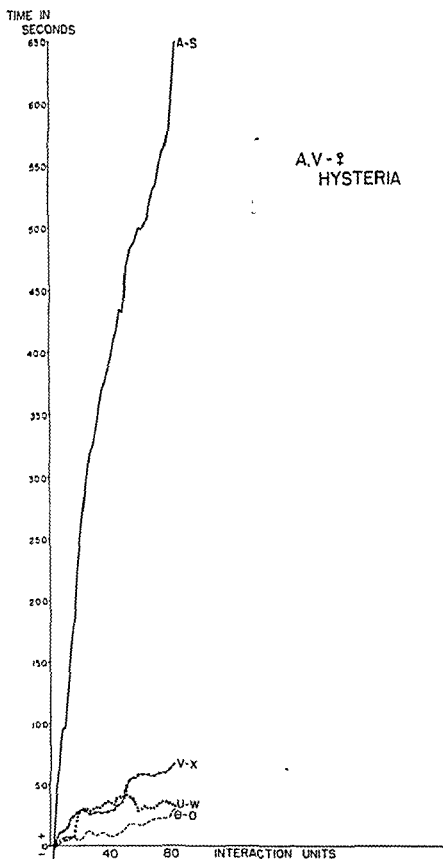


Fig. 3.

The individual who gives the interaction of Fig. 1 might well show a completely different picture in a different situation. However, in most any situation, the patient under discussion would give a picture similar to Fig. 2. She

is a patient whose chief complaints were of an allergic nature, migraine, angioneurotic edema, and chronic vasomotor rhinitis. She also had complaints of diarrhea, epigastric pain, palpitations, and neuralgia. The presence of allergens was demonstrated by skin tests, but because of the multiplicity of her complaints, she was treated on the psychiatric ward.

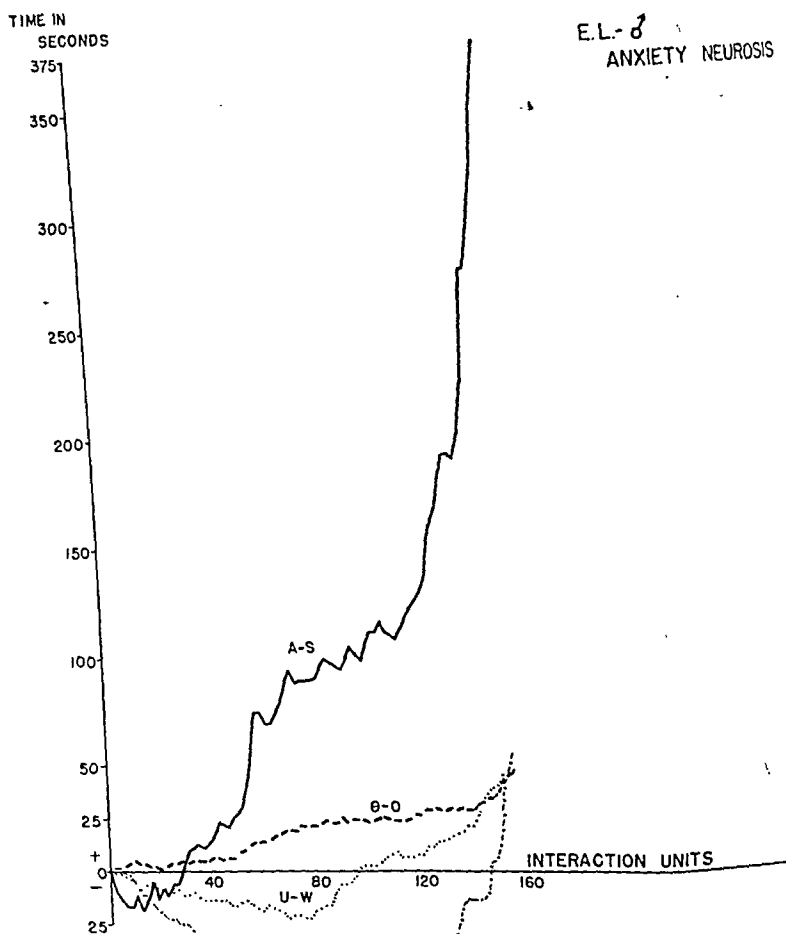


Fig. 4.

This patient had a domineering mother, an invalid father, and two marriages which ended in divorce. The first marriage was prompted by a desire to escape from the dominance of the mother and by it the patient had a son who has since been committed to an institution with the diagnosis of dementia praecox. The patient had undergone an appendectomy and a panhysterectomy. Since the last divorce, she had lived alone in an apartment and practiced an engineering profession until entry into the hospital. Her illness was of a chronic nature, and her improvement was only slight during her hospital stay. The diagnosis was migraine, hypochondriasis, and neuralgia of undetermined type.

From her earliest contacts with people this patient had undergone di-

turbances to her equilibrium and emotional strain. Through many years of failure to achieve proper adjustment in her interpersonal relations, this patient seems to have developed an improper functioning of her autonomic nervous system, manifested by her varied symptoms.

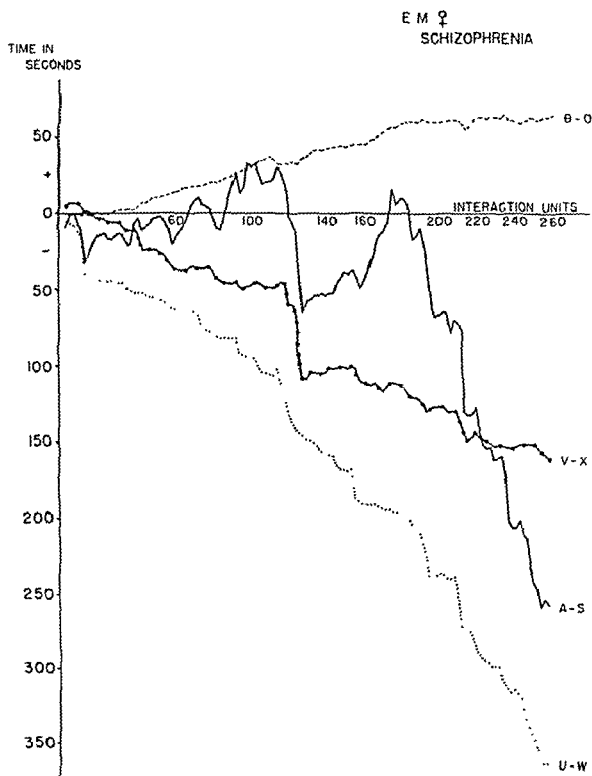


Fig 5.

Fig. 3 represents a case of hysteria. The cases so far examined by the interaction chronogram technique show a very steep activity curve; this means that they talk almost continuously. The horizontal position of the doctor's adjustment curve (V minus X) in the first half of the curve indicates that he was able to adjust well to the patient, but as the interview progressed, his adjustment curve rises steeply, illustrating that he was unable to maintain this satisfactory adjustment and began to interrupt the patient frequently.

Fig. 4 illustrates the typical interaction of a patient with an anxiety neurosis. In contrast to the hysteria presented before, the activity rate is in no way as pronounced, although in the latter part of the interview there is a short spurt of very high activity. The patient's adjustment curve has a definite negative slope for the first half of the interview, showing that until the latter part, there is a marked hesitancy and failure to respond when the doctor stops talking.

### LEVEL OF ADJUSTMENT

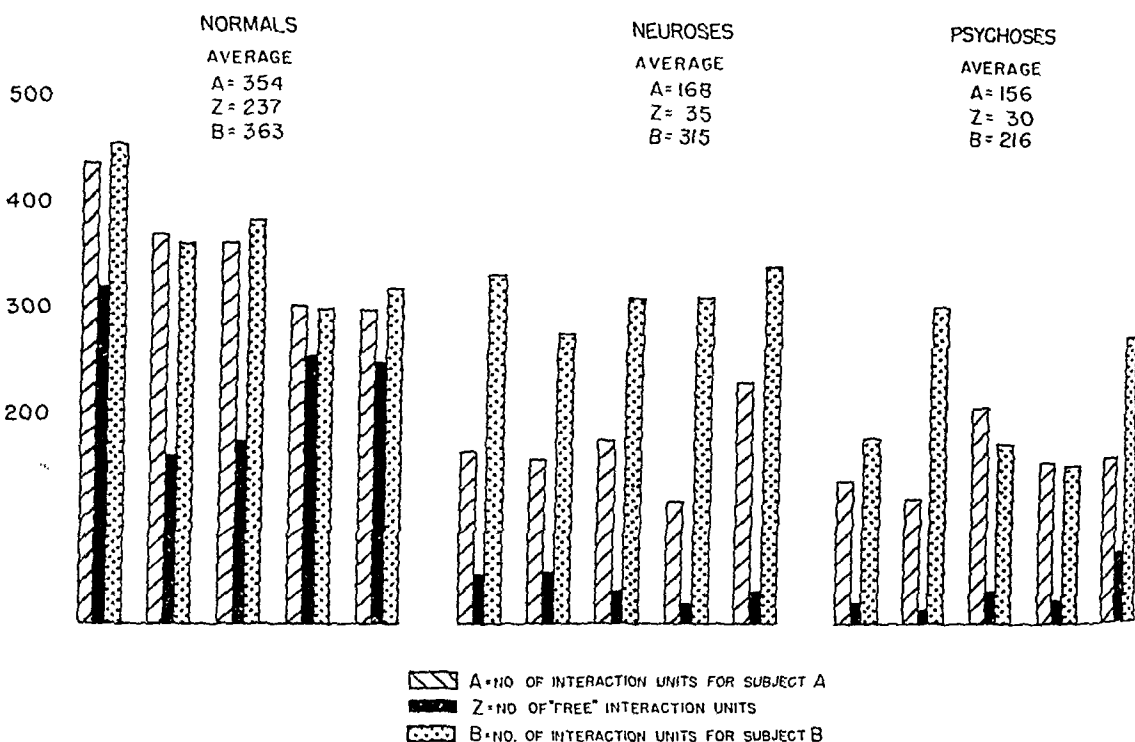


Fig. 6.

Other psychoneuroses, such as obsessions and depressions, also present more or less characteristic interaction rates, and examples have been published elsewhere, together with instances of the several psychoses.<sup>19, 21</sup> One psychosis is represented here for comparison, namely a case of schizophrenia. Notice in Fig. 5 the characteristic alternations between great activity and inactivity, together with extremely pronounced failures to respond in the patient's adjustment curve. The doctor's adjustment curve (V minus X) is interesting, since it shows the use of two different techniques in an attempt to bring about adjustment: waiting for the patient to start in again, and trying to adjust to the patient. Both had no effect.

Probably the most important criterion in the interaction chronogram for differentiating normal individuals from those who are suffering from psychogenic disorders lies in their adjustment curves. Perfect adjustment is defined

as the absence of interruptions and failures to respond. The degree of adjustment can be easily determined by comparison of the number of interaction units free of interruptions or failures to respond with the total number in the interview. Normally the proportion of free units to the total number of interaction units is high. In the individuals suffering from psychogenic disorders, this proportion is very low. Fig. 6 shows the result of calculating this proportion for five normal, five neurotic, and five psychotic patients.

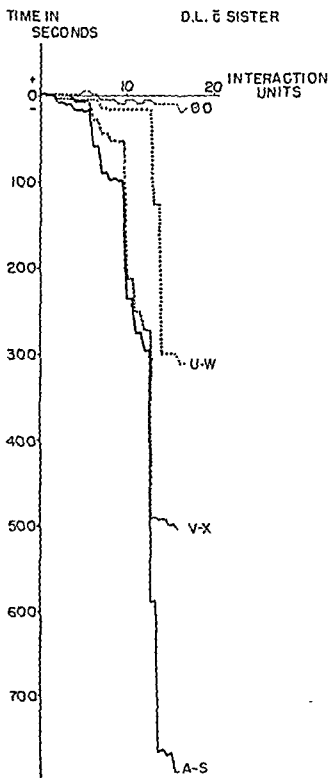


FIG. 7.

Not only does the measurement of interpersonal relations through the use of the Interaction Chronograph seem to be a help to the physician to determine when he is dealing with psychosomatic, psychotic, and psychoneurotic disorders, but it also may afford him an opportunity to treat the milder cases

manipulating the environment in order to help the person re-establish his equilibrium. A case in point is presented in Figs. 7 and 8. They are clinical records of the interaction (1) between the patient, a boy of sixteen, and an older sister, and (2) between the boy and the physician. This patient is the youngest of eight children, the oldest aged thirty-seven, the next to youngest being twenty-one. His mother died during his infancy, and he spent some childhood years in a foster home and an orphanage. When he was ten years old, he returned to live with his family, the other members of which were much older than he. At the time of the interview, he was living with his father, aged sixty, and three siblings. He was suffering from chronic fatigue and fainting spells, which had developed following a traumatic head injury.

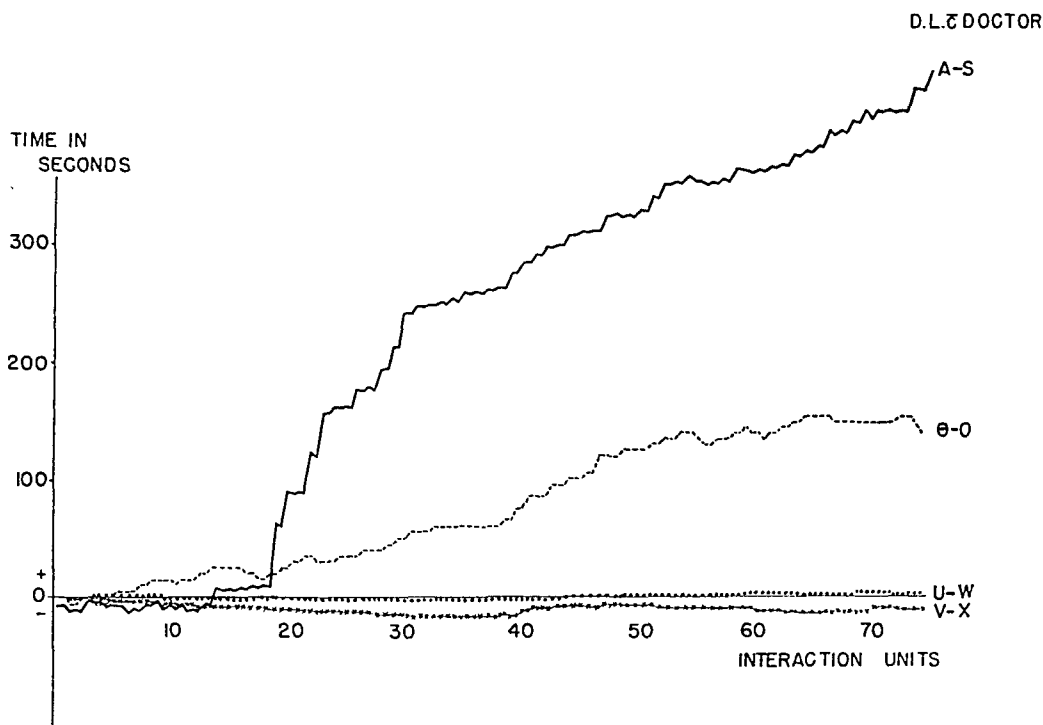


Fig. 8.

No neurologic lesion could be demonstrated. Fig. 7 pictures his interaction with his sister while the two were waiting to see the doctor. During the twenty-minute observation period, he was silent the greater part of the time. He did not respond to talking on his sister's part, as is evidenced by the negative slope of the U minus W curve. His initiative curve also descends. The descent of the V minus X curve indicates that during the few times the patient did respond to his sister, she failed to continue the conversation. Thus, it appears from our measurements that he has a very weak bond for interaction with his sister despite the fact that she is the closest of the family to him in age. Social service studies indicated that his bonds with other members of the family were no stronger. The patient was, therefore, unable to maintain an equilibrium at home, and since he had no outside activities, he

was unable to compensate for it elsewhere. The sister likewise is unable to maintain her equilibrium by interacting with her brother, but in the family group she has strong bonds with the older members of the family.

In Fig. 8 the patient interacts quite normally with the doctor. In fact, he has positive slopes in his activity rate; this indicates that for equilibrium he would require a moderately high amount of interaction. The treatment for the patient obviously involved changing his environment so that he could build up relations in which the interaction was sufficient to maintain his equilibrium. He, himself, expressed a desire to leave home, but his father forbade it. He was helped to find a job away from home and when last heard from was well; his symptoms had entirely disappeared.

#### CONCLUSION

The advantage to the physician of these methods of studying human interaction should be clear. They provide an objective set of criteria which can be used to deal with the problems of interpersonal relations. By the use of such criteria in describing a patient's behavior and by obtaining a careful history, it is possible to begin to formulate interpersonal factors in a systematic manner. The family doctor has long been aware of these factors by knowing his families most intimately.

The Interaction Chronograph should be of use to psychiatrists and clinicians in investigative work especially, but an adequate understanding of the information brought out in these studies should enable the clinician to understand more fully his patients' personalities and to prescribe appropriate therapeutic measures, even though he does not actually use the interaction chronogram. This method of studying interpersonal relations is relatively new and, of course, does not give an inclusive measure of physiologic and psychologic "constitutions." We are dealing with a most complex mechanism, and this method is only one approach.

In this connection Stanley Cobb has said, "Studying the 'whole individual' would have to be a sort of armchair contemplation that would lead nowhere. Likewise, no student ever completed a description of the 'total situation' of another person. It cannot be done. What one can do is this: approach the study of diseases recognizing that every human being is a whole individual and not the mere sum of his parts. That is something different, and only thus can one study the parts intelligently."<sup>20</sup> That very important part of an individual, his personality, the way he reacts with other people, can be studied objectively by the method which we have described. The concept of interaction and the application of physiologic thought and method to the study of personality appears to us to provide both the physician and laboratory worker with a valuable tool for studying human behavior.

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# THE EFFECT OF SUBCUTANEOUS INJECTION OF URINE AND URINARY EXTRACTS FROM RHEUMATOID PATIENTS INTO RATS\*

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WHILE the etiology and pathogenesis of rheumatoid arthritis remain unknown, its clinical features, constituting a well-recognized disease entity, are more sharply defined. One of the principal characteristics of the illness is plurisystemic involvement. Both the clinician and the pathologist know that besides the articulations and related skeletal structures, pleura and pericardium are at times affected. If recent observations are borne out, the endocardium may likewise be a site of injury in the disease. Adenopathy is frequent, and hepatomegaly and splenomegaly are occasional findings in rheumatoid arthritis. While some of the common symptoms and signs, as paraesthesias, vasomotor disturbances, diarrhea, and cachexia, may be secondary phenomena, the occurrence of rheumatoid nodules in the serosa, pathologically identical with those found in the subcutaneum at other locations, suggests a fairly typical reaction on the part of several bodily systems to one or more disseminated disease agents. The thought that such a substance might be present in the circulating blood stream and excreted in the urine of rheumatoid patients has undoubtedly motivated previous investigations, but we found no reports in the available literature indicating that the injection of urine or urinary extracts from such patients into laboratory animals had been undertaken. The following experiments were therefore done, mainly in an attempt to fill the gap and with the realization that a negative result would be inconclusive, both because the methods used in obtaining the injected material may alter the substance sought and the receiving animal may not be susceptible to its action.

## MATERIALS AND METHODS

Twelve adult male albino rats were used. Urine was pooled from only rheumatoid patients with active disease as gauged by clinical evidence and sedimentation rate. Collection of the first morning voiding into clean glass receptacles was followed by immediate processing. A small part of the urine was passed through a Seitz filter into a sterile container, and 1 c.c. of the fresh material was injected into the subcutaneum of the abdomen of 3 rats 4 times a week.

One half of the remaining urine (about 1500 c.c.) was extracted with 70 per cent alcohol after adsorption with Kaolin, following a modification of the

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procedures used by Houssay and Biasotti,<sup>1</sup> and Wearn, Miller, and Heinle.<sup>2</sup> The extract was evaporated under vacuum, the residual taken up in sterile saline to make 5 c.c., and adjusted to a pH of 7.4. One c.c. of this preparation, corresponding to 300 c.c. of urine, was injected into 3 rats in the same way as was the fresh sterile urine. The remaining urine (about 1500 c.c.) was extracted 3 times with chloroform in a separatory funnel. The extract was evaporated under vacuum and the residual taken up in sterile sesame oil to make 5 c.c. One-half c.c. of this preparation, corresponding to 150 c.c. of fresh urine, was injected into 3 rats as above.

### RESULTS

One rat receiving injections of straight urine died of lobar pneumonia after the thirty-first inoculation. All others tolerated treatment for four months, after which time they were sacrificed. The other 2 animals in the first series and those receiving the alcoholic extract lost weight and showed evidence of failing health, such as deterioration of coat, apathy, and irritability. The 3 animals treated with the chloroform extract in sesame oil appeared to suffer no ill effect at all. None of the 12 animals developed any articular lesion. Immediately upon death, autopsies were performed. Grossly and microscopically, the joints were normal. The other skeletal parts were likewise examined, as were the viscera. Such changes as were found in the internal organs were apparently nonspecific and inconstant.

### SUMMARY AND CONCLUSIONS

1. Twelve rats were injected subcutaneously with unprocessed urine and alcohol and chloroform extracts of urine from patients with active rheumatoid arthritis.

2. The animals tolerated the procedure well and developed no significant lesions.

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## ACTIVE AND PASSIVE IMMUNITY IN EXPERIMENTAL HEMOPHILUS PERTUSSIS INFECTION IN MICE\*

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IN THE past several years we have isolated several fractions from *Hemophilus pertussis* bacilli and from the fluid in which these organisms grew. It became necessary to test the efficacy of these fractions as active immunizing agents and to investigate their ability to produce specific antibodies. Before anything could be done, however, we had to have a test, a standard, an accurate measure of protection, which could be repeated and demonstrated at will under similar circumstances.

Our first problem then was to see if we could consistently produce the experimental disease. Only after this was done would it be expedient to try and demonstrate immunity.

At the time these experiments were performed, there were three methods in use to produce experimental pertussis, those of Silverthorne,<sup>1</sup> Bradford,<sup>2</sup> and Lawson.<sup>3</sup>

### METHODS OF INJECTING MICE

1. *Silverthorne's Method.*—Silverthorne incubated phase I organisms for 48 hours, scraped the growth, suspended it in saline, and standardized the solution at 48 billion organisms per c.c. by bacterial count. We will refer to phase A, the disease-producing state of the organism's existence, as distinguished from phase B, the nondisease-producing state. This would correspond to phases I and IV of Leslie and Gardner, respectively. It is impossible, in our opinion, to distinguish accurately phases other than these two.<sup>4</sup> The organism suspension was then added to 5 per cent swine gastric mucin, prepared according to the method of Mishulow, Klein, Liss, and Liefer<sup>5</sup> in a 1:10 saline dilution and injected intraperitoneally into mice. The mucin was designated as #1701-W and obtained from Wilson & Co.

*Results.*—All of the Swiss mice (100 per cent) injected with the organism-mucin suspension died within 48 hours, while 4 or 26.6 per cent of the controls died. Thus, all the positive controls died. The number of deaths that occurred in the negative control group was not so high that adjustments could not have been made for this error.

This method has the disadvantage of introducing an abnormal variant, i.e., the mucin; it also has the disadvantage that the injection is made intraperitoneally, an unnatural portal of entry (Experiment I, Table I).

2. *Bradford's Method.*—A long incision is made over the ventral surface

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of the neck, the muscles are retracted, the trachea is pulled into view with a bent probe, and 0.05 c.c. of organisms standardized at 10 billion organisms/1 c.c. injected between rings directly into the lumen of the trachea. The edges of the skin are then approximated and held in place by collodion.

Phase A *H. pertussis* organisms were grown on Bordet-Gengou media for 72 hours and scraped off into 0.85 per cent saline solution. Thirty Swiss mice were injected with 0.05 c.c. of the organism suspension. Eleven control animals were given 0.05 c.c. of saline.

*Results.*—Only 83.3 per cent of the infected animals died (Experiment II, Table I). Although Bradford's is a good method, its disadvantage is that an operation has to be performed and an anesthetic given in order to introduce the organisms.

3. *Lawson's Method.*—This worker anesthetized his animals, held them upright, pulled the tongue forward, occluded the nostrils by pinching to insure aspiration, and injected 0.05 c.c. of a standardized suspension of organisms by syringe through a J-shaped needle over the posterior portion of the tongue.

Phase A *H. pertussis* organisms were grown on Bordet-Gengou media for 72 hours, scraped off, emulsified in normal saline, and standardized by bacterial count to 1 billion organisms/0.05 c.c. dose. Eighty Swiss mice were used, 50 of which were injected.

TABLE I

EXPERIMENT	NUMBER ANIMALS	INJECTION	RESULT		NUMBER ANIMALS	INJECTION	RESULT	
			DIED	PER-CENTAGE			DIED	PER-CENTAGE
I	30	Silverthorne	30	100	15	Mucin	4	26 $\frac{2}{3}$
II	30	Bradford	25	83.3	11	Saline	3	27
III	50	Lawson	45	90	30	Saline	3	10
IV	50	Lawson	49	98	30	Saline	1	.03 $\frac{1}{4}$

*Results.*—There was a definite response. Ninety per cent of the injected animals died, and only 10 per cent of the controls (Experiment III, Table I).

The anesthesia caused some deaths. Since the procedure of insufflation was painless, anesthesia was not employed subsequently.

We next tried to determine whether there was any difference in reaction between Swiss and white mice.

Eighty white mice were used, 50 of which were injected, 30 being controls. The results were conclusive. Ninety-eight per cent of the injected and 3 per cent of the controls died (Experiment IV, Table I).

*Conclusion.*—Since a high mortality rate consistently followed, since the rates of death following the use of this were as high if not higher than with other methods, since the way in which the disease was produced was a natural one, since there were fewer variabilities to consider, since there was no pain and since anesthetics could be avoided, it was decided to use Lawson's technique with the modification mentioned, i.e., without the anesthetizing process. Since, also, there was but little difference between Swiss and white mice, the latter were used exclusively in the subsequent experiments.

#### ACTIVE AND PASSIVE PROTECTION EXPERIMENTS

One infers from reading the literature that phase A (phase I) vaccine is an efficient active immunizing agent against the homologous phase organism.

It was fundamental for us to know whether we could actively immunize mice, since if this were possible, the immunizing value of other fraction antigens could be compared with it.

### *Experiment V Active Immunity.*—

#### METHODS AND MATERIAL

Both phase A (lyophilized organisms 5) and phase B (#778) *H. pertussis* were grown for 72 hours, the former on Bordet-Gengou blood agar media and the latter on brain veal agar. The organisms were suspended in 1:10,000 merthiolate solution and standardized at 10 billion/c.c. by bacterial count. The vaccine stood for 8 to 12 days, after which it was tested for sterility. It was necessary to add 0.2 per cent formalin to the phase A bacteria, since the organisms were still alive. Subsequent recultures were sterile. The mice to be actively immunized were injected subcutaneously at weekly intervals with doses of 0.2 c.c., 0.2 c.c., 0.3 c.c., and 0.5 c.c. vaccine, a total of 1.2 c.c. There were 10 groups of animals in this experiment.

*Group I* was composed of 66 mice which were immunized with phase A organisms in the usual way. Only 27 animals withstood the immunization process.

*Group II*, 58 animals, was immunized with a vaccine standardized at 1 billion phase A organisms per c.c. in the same dosage as Group I; 45 survived the immunization process.

*Group III*, 63 animals, was injected with a phase B vaccine standardized at 10 billion B organisms/c.c.; 47 animals survived.

*Group IV*, Phase A organisms were standardized at 10 billion organisms/c.c., and washed with sterile distilled water 5 or 6 times until clear. The washings were concentrated to 75 per cent of the original volume in the lyophile apparatus and preservative was added. Sixty-one animals were immunized with the usual 5 weekly doses. Despite the number of times centrifuged, at least 1 billion organisms/c.c. were still present by actual bacterial count. Forty-six animals survived the immunizing procedure.

*Group V*, 64 animals, was injected with the washed organisms which remained from Group IV. The organisms were standardized at 10 billion/c.c. and the usual amounts injected. Thirty-six animals survived.

*Group VI*, 67 animals, was immunized with washings of phase A organisms processed in a Sharples high-speed centrifuge machine; 26 survived.

*Group VII*: We have been able to isolate the fraction causing agglutination and to obtain it in powdered form. It was used as an antigen in the same amounts as other antigens used in this work.

*Group VIII*: Agglutinin antibody serum was produced in rats by injecting the pure powdered agglutinin. The serum was used as a passive principle in the usual protective type of experiment.

*Group XI*: Fifty animals were used as positive controls receiving virulent organisms without previous protection.

*Group XII*: Five animals received only saline and were used as negative controls.

All the animals in each group received 0.1 c.c. of phase A organisms standardized at 1.5 billion/0.1 c.c.

*Results.*—Our results were obvious. None of the immunization procedures employed established any striking protection. This was surprising since we had felt that phase A organisms would give us some protection. We did not expect phase B organism vaccines to protect, since they had never protected before in any of our previous experiments except against their homologous strains.

If our conception of initial sensitization is correct, then the reaction with the agglutinin fraction was as expected.<sup>6</sup> There would be an accelerated production of the disease and the animal would die more quickly than comparable controls. This is what happened.

*Experiment VI Active Immunity.*—Because the phase A organism used did not establish protection beyond that which followed other types of antigens used, it was decided to repeat the experiment using a different and more highly virulent organism.

In the last experiment during the time interval between immunization and injection, the organisms might have lost some of their phase A characteristics and be proceeding toward the phase B stage. The organisms were kept in the virulent state by lyophilizing them.<sup>7</sup> This procedure did not modify the virulence of *H. pertussis*, for after processing, 50 of the 50 mice were killed in 72 hours by the standardized dose. The organism was again tested for virulence a few months later, and it still caused a 100 per cent mortality rate.

#### METHODS AND MATERIALS

The antigens used were phase A organisms standardized at 10 billion/c.c., phase A organisms standardized at 1 billion/c.c., washings from phase A organisms, the washed organisms and again the agglutinin material, together with negative and positive controls.

The dosage and the methods used were the same as in the previous experiment.

*Results.*—The results were clear-cut. The negative controls all lived; the positive controls all died. Again there was but little protection brought about by vaccination with 10 billion phase A organisms/c.c.; again it was demonstrated that the agglutinin antigen actually hastened the death of the animals (Experiment VI, Table II):

*Experiment VII Active and Passive Immunity.*—These two previous experiments, although clear-cut, were at variance with our ideas of what should happen when phase A organisms were used as the antigen in mouse protection tests. Accordingly, we repeated the experiments, using a new strain. We did not use the Group IV antigen (antigen washings obtained from washed organisms), but we did try to demonstrate the protective effect of hyperimmune serum<sup>8</sup> (Group IX). This serum was obtained from an individual who had been injected subcutaneously with 2 c.c. of Sauer's vaccine twice a month, from June to November of 1940.

For a third time, our conclusions were the same as before. Phase A antigen did not protect. The hyperimmune serum gave protection, since only 30.6 per cent of the animals died as compared to 85.7 per cent of those protected with phase A vaccine and 100 per cent of the positive controls.

*Experiment VIII Active and Passive Immunity.*—The three experiments

TABLE II  
PROTECTION EXPERIMENTS

ANTIGEN OR PASSIVE IMMUNE PRINCIPLE	EXP. NO.	STRAIN USED	NO. MICE USED	TOTAL DEAD				GRAND TOTAL DEAD				GENERAL AVERAGE	
				1-7 DAYS	%	8-14 DAYS	%	15-21 DAYS	%	NO.	%	TOTAL INJ.	%
1. Phase A 10 billion/c.c.	5	12	37	25	85.1	24	88.8	-	-	24	88.8		
	6	12	92	75	81.6	79	85.8	79	85.8	79	85.8		
	7	12	49	40	81.6	42	85.7	42	85.7	42	85.7		
	8	12	142	86	60.6	99	69.7	105	73.9	105	73.9	310	80.6
2. Phase A 1 billion/c.c.	5	12	45	38	84.4	41	91.1	-	-	41	91.1		
	6	12	84	74	88.0	74	88.0	75	89.2	75	89.2		
	7	12	73	65	89.0	68	90.4	66	90.4	66	90.4	202	90.0
3. Phase B, 10 billion/c.c.	5	12	47	47	100	-	-	-	-	47	100.0	47	100.0
4. Phase A washings	5	12	46	33	71.7	35	76.1	35	76.1	35	76.1		
	6	12	93	76	81.7	81	87.1	81	87.1	81	87.1		
	7	12	74	63	85.1	65	87.8	66	89.1	66	89.1	213	85.4
5. Phase A washed organisms	5	12	36	31	86.3	22	61.1	-	-	22	61.1		
	6	12	93	82	88.2	85	91.3	85	91.3	85	91.3	126	84.1
6. Shorples washings	5	12	26	23	88.4	25	96.2	-	-	25	96.2	26	96.1
7. Isolated Agglutinin	5	12	27	26	96.3	27	100	-	-	27	100		
	6	12	67	67	100.0	-	-	-	-	67	100	94	100
8. Agglutinin Serum	5	12	20	18	90.0	20	100	-	-	20	100	20	100
9. Hyperimmune Serum	7	12	60	12	20	15	25	17	28.3	17	28.3		
	8	12	100	31	31	36	36	42	42.0	42	42.0	160	30.6
10. Lilly Serum	8	12	78	48	61.6	53	67.9	56	71.7	56	71.7	78	71.8
11. Lederle Serum	8	12	93	34	36.6	39	41.9	42	45.1	42	45.1		
	9	7	90	21	23.3	32	35.5	39	43.3	39	43.3	183	44.2
12. Positive Controls	5	12	50	50	100	-	-	-	-	50	100		
	6	12	50	47	94	50	100	-	-	50	100		
	7	12	116	111	95.6	116	100	-	-	116	100		
	8	12	100	87	87	92	92	97	97	97	97	316	99.0
13. Negative Controls	5	12	5	-	-	-	-	-	-	0	0		
	6	12	50	-	-	-	-	-	-	0	0		
	7	12	116	5	4.3	22	18.9	28	24.1	28	24.1		
	8	12	100	6	6.0	13	13.0	19	19.0	19	19.0	316	47
14. Human Serum, Admission	8	12	97	62	65.0	70	72.1	73	75.2	73	75.2	97	73.2
15. Convalescent Serum, Discharge	8	12	90	39	43.3	49	54.4	55	61.1	55	61.1	90	61.1



previously described did not show that phase A organisms actively immunized mice so well as has been reported. It was thought wise to do the active immunizing experiments again with larger numbers of animals and to compare the results with those obtained by protecting with hyperimmune serum (Group IX, Table II), with serum obtained from patients at the height of their disease (Group XIV), with serum obtained from the same patients later during their convalescence (Group XV), and with commercial antipertussis serums (Groups X and XI).

There is little mention in the literature of what happens when mice are being immunized with the phase I organism. Our experience has been that nearly 50 per cent or more of the animals die during this process. In the present experiment, we began to immunize 300 animals with phase A vaccine; only 142 survived.

*Results.*—The results from active immunization of this group of 142 mice were the same as with the three previous experiments when the animals were tested with a virulent dose of pertussis.

There were a lesser number of deaths following the use of human convalescent serum as a passive principle as compared to that number which followed the use of serum obtained from patients still in the incubation period (61 per cent and 75 per cent, respectively). One might say that there was a definite trend in the right direction, but perhaps the protection factor had not had time to mature and the serum was obtained too early.

One of the commercial antitoxins was in horse serum and gave but little passive protection. The commercial rabbit antipertussis serum showed definite evidence of protection. Hyperimmune serum again showed some protection, only 42 per cent of these animals dying as compared with 99 per cent of the controls.

*Experiment IX.*—The rabbit serum which was used in the above experiment was again tried in an isolated passive protection experiment. Only 39 of 90 such animals or 43.3 per cent died. This confirmed our previous results.

#### COMMENT

One is at a loss to understand why so little protection was obtained with phase A vaccine. It was not due to a paucity of animals. It might be said that we had more than a minimum lethal dose. Even were this true, there should have been a trend towards protection, a trend which at no time was obvious, except as indicated.

#### CONCLUSION

1. Under the conditions of these experiments, using *Lawson's method* to produce the disease, freshly isolated phase A *H. pertussis* organisms gave but slight protection to white mice when an attempt was made to immunize them actively with those organisms. The mortality rate was 80.6 per cent as compared with 99 per cent in the controls.

2. Under the conditions of these experiments, antigens obtained from washings of the organisms, the washed organisms themselves, phase B organisms, and the lesser dilutions of phase A organisms, gave no protection in white mice when used in an attempt to produce active immunity.

3. Only slight passive protection was conferred by convalescent human serum; a slight degree of protection was given by one commercial horse anti-serum, since only 71.8 per cent of the animals died as compared with 97 per cent of the controls. Better protection was given by another antipertussis serum (rabbit) with a mortality of 44.2 per cent; the best protection, however, was obtained when we used human hyperimmune serum (30.6 per cent mortality rate).

4. Neither the pertussis agglutinin fraction nor the agglutinin fraction anti-serum protected white mice. In fact, these materials accelerated the death of these animals; this is possible evidence that a phase of the disease is one of sensitivity.

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## ANEURYSMS OF THE VERTEBRAL ARTERIES; A CONSIDERATION OF THEIR ETIOLOGY\*

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THE frequency of intracranial aneurysms is indicated by the following observations: Fearnside<sup>1</sup> found 44 cases in a group of 5,432 autopsies; Osler<sup>2</sup> observed 12 in 800 autopsies; Pitt<sup>3</sup> found 19 in approximately 9,000 autopsies; and Conway<sup>3</sup> found 43 cases of undoubted cerebral aneurysms among 6,325 sections. The vessels in the intracranial cavity have more aneurysms upon them than any other group of arteries of corresponding size in the body. The thinness of the wall of these arteries and the absence of supporting tissue may be factors in the frequent development of these lesions.

Bradford,<sup>4</sup> in discussing the frequency of aneurysms on the cerebral vessels, says: "One cannot help thinking sometimes that the incidence of aneurysms of the cerebral vessels must be linked to the difference in the physiology of the cerebral circulation as compared with such a circulation as that of the viscera. The cerebral arteries are engorged passively whenever the tension rises in the systemic vessels . . . they are passively distended as the result of the increased aortic pressure which is transmitted to them through the column of blood."

The distribution of aneurysms upon the different intracranial arteries is significant in considering the etiology of the process. McDonald and Korb<sup>5</sup> assembled from the literature 1,125 cases of intracranial aneurysms published between 1761 and 1938. The largest number of aneurysms in this group occurs upon the cerebral arteries; the second most frequent site is upon the internal carotids. The smallest number of aneurysms is associated with the vertebral arteries.

Arteriosclerosis according to Dial and Maurer<sup>6</sup> undoubtedly plays the major role in the formation of intracranial aneurysms. Seven of their thirteen cases are considered to be associated with arteriosclerosis. The observation of McDonald and Korb<sup>5</sup> that "in 49.5 per cent of the 572 cases in which the vessels were described there were sclerotic arteries" may indicate the role of arteriosclerosis in the formation of these aneurysms. Keegan and Bennett<sup>7</sup> express the opinion, however, that arteriosclerosis is rarely the cause of cerebral aneurysms. Lebert,<sup>8</sup> as early as 1866, says, "Calcifying atheroma is rarely found in cases of cerebral aneurysm and is not an important cause of their formation." Hoffman<sup>9</sup> in 1894 likewise concluded from a study of 78 cases that atheroma was not a common cause in the formation of aneurysms; however, atheroma and a heightened blood pressure were important factors in the causation of rupture. In a large group of cases of intracranial aneurysms Parker<sup>10</sup> could find no evidence of arteriosclerosis anywhere in the body.

\*From the Department of Pathology, University of Tennessee.  
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Syphilis apparently plays an insignificant role in the etiology of intracranial aneurysms. Sands<sup>11</sup> says that "it is remarkable that syphilis does not play any part in the formation of cerebral aneurysms." Symonds<sup>12</sup> is of a similar opinion and says, "Contrary to former belief, it [syphilis] has been shown to be a rare cause of intracranial aneurysms." Turnbull's<sup>13</sup> observations on syphilitic arteritis are important. "When the smaller muscular and elastic arteries are the seat of syphilitic inflammation endarteritis is usually a marked feature, the lumen becoming almost obliterated. It is clear that in such a condition aneurysmal dilatation is excluded. In some cases of intense inflammation with necrosis the adventitia and media become greatly weakened before the intima has greatly thickened; in such cases aneurysmal dilatation might occur but in my experience such intense inflammation has led to rupture of false aneurysm and not to the true aneurysm." It does seem that these aneurysms would be observed more often if they should follow arteritis, since syphilitic and tuberculous infections are so frequent in the cranial cavity, and usually the blood vessels are involved in the inflammatory process. McDonald and Korb<sup>5</sup> found evidence of syphilis in the cerebral blood vessels in 32 out of 368 cases of aneurysms which they collected from the literature.

Systemic infections and vegetative endocarditis with septic emboli may produce some of the aneurysms that occur in the cranial cavity. Fearnside's<sup>1</sup> had 13 cases that resulted from an acute infectious process in a group of 44 intracranial aneurysms. McDonald and Korb<sup>5</sup> found the pathologic lesions to be embolic in 62 out of a group of 368 cases of intracranial aneurysms which they collected from the literature.

Many intracranial aneurysms occur in individuals that show no clinical signs of either vascular or cardiac diseases; neither do they show any signs of any causal agent as being responsible for the aneurysms. In such cases Conway<sup>7</sup> says, "I must consider that these aneurysms are of congenital origin." Dial and Maurer<sup>8</sup> found it difficult to evaluate the role which anomalies and congenital aneurysms play in the formation of intracranial aneurysms. Some investigators consider that congenital defects are significant in the frequent development of these aneurysms. Forbus<sup>14</sup> has shown very nicely that the media in the wall of the cerebral arteries may fail to unite in the acute angle formed at a bifurcation. This defect may not always lead to the development of an aneurysm; however, its presence predisposes to the development of an aneurysm at this site. Turnbull<sup>13</sup> concluded that diffuse dilatation due to medial degeneration "is frequently found in the muscular arteries including the cerebral."

It is certainly difficult to evaluate the role that trauma may play in the etiology of intracranial aneurysms.<sup>6</sup> Ayer<sup>15</sup> has suggested that an intracranial aneurysm can be a sequel to a head injury in cases in which the membranes are torn. In the process of healing these membranes form adhesions with the periarterial tissues and torsion results in the vessels. These serve to form pouches, and in this way the wall of the vessels becomes weaker. Turnbull<sup>13</sup> and Fearnside's<sup>1</sup> however, do not consider trauma to play any role in the formation of intracranial aneurysms. Parker<sup>10</sup> does not think that trauma is usually a cause of these aneurysms, although it may provoke rupture of the distended sac.

In considering the etiology of intracranial aneurysms it seems important

to study separately the aneurysms associated with the different arteries or groups of intracranial arteries and to consider the location of the aneurysms with regard to bifurcations and branches. In a review of the literature it would seem that the data are conclusive on the development of aneurysms at the points of bifurcation. It is likewise obvious that a mycotic aneurysm may develop either as a result of a localized area of arteritis or as secondary to an infected embolus.

Only 68 aneurysms have been reported as occurring on the vertebral arteries before 1938.<sup>5</sup> Since this time, two additional cases have been reported by Bassae.<sup>17</sup> In the excellent review by McDonald and Korb<sup>5</sup> they found that the right vertebral artery was the site of the aneurysm in 23, and the left in 36 cases, while both vertebral arteries were involved in two cases. The involved artery was not given in seven of the cases. These aneurysms occurred in 40 males and in 22 females; the sex was not recorded in 6 cases. The age distribution is very significant in considering the etiology of vertebral aneurysms. The following is the age at which they were found in a series of cases reported by McDonald and Korb<sup>5</sup>:

0- 9 years	2 cases	50-59 years	10 cases
10-19 years	3 cases	60-69 years	9 cases
20-29 years	2 cases	70-79 years	3 cases
30-39 years	7 cases	80- years	0 cases
40-49 years	11 cases	Age not recorded	21 cases

These data show that 44.7 per cent of the vertebral aneurysms occur between the ages of 40 and 60 years, 78.7 per cent between 30 and 80 years of age. Of the 68 cases of vertebral aneurysms reported by McDonald and Korb,<sup>5</sup> 44 had ruptured, while 17 were unruptured; these data were not given in 7 of the cases. Insufficient data are given on the aneurysms occurring in the younger age group to indicate their possible etiology.

The following two cases have been observed by us and illustrate some of the factors to be considered in the etiology of vertebral aneurysms.

CASE 1.—A. R., a colored male 49 years of age, was in coma when brought to the hospital. A relative said that she was awakened at 2:30 A.M. by the patient "yelling." He was unable to speak at this time. The patient's neck was stiff, the pupils were fixed and did not react to light when admitted twelve hours later. The right pupil was larger than the left. The discs were pale, and numerous small hemorrhages were present. Kernig's and Brudzinski's signs were questionably positive. The abdominal and patellar reflexes were absent. The spinal fluid pressure was 240 mm. of water, and the fluid was bloody. The Queckenstedt test was normal. The spinal fluid chloride was 760 mg. per 100 c.c., and the sugar, 30 mg. per 100 c.c. The blood pressure was 170/110, the temperature was 100.4° F., the pulse was 100, and the respirations were 40. The Kahn reaction on the blood was negative, although the Wassermann reaction on the spinal fluid was positive.

Apparently the patient could neither understand the spoken word nor speak during the time he remained in the hospital. His condition improved shortly after hospitalization, and he did recognize members of his family. He again became comatose and gradually grew weaker until his death on the tenth day following admission. The temperature during hospitalization varied between 99 and 104° F.

During the past three years the patient has had "kidney trouble," the characteristics

of which are not given. Seven months before death he "fell out." There is no history relative to this episode.

*Pathologic Examination.*—Only the pertinent findings are recorded. The autopsy was done one hour following death. There was considerable blood in the subarachnoid space, especially over the occipital and parietal lobes. In the area of the medulla the blood was clotted and appeared older than that in the other portions of the brain. A small amount of blood was present in the tip of the lateral ventricles. In serial sections through the brain several small infarcts were found, one of which was a recent lesion. The source of the subarachnoid hemorrhage was an aneurysm located on the lateral portion of the right vertebral artery approximately 2 mm. proximal to the basilar artery (Fig. 1). This aneurysm measures 1.5 cm. in diameter and was partially filled with a thrombus. The wall was thin and at one point it had ruptured. Old blood was present in the tissues around the pons and the medulla.

There was considerable atherosclerosis in the left vertebral artery and in the basilar; however, there was only a minimal amount of sclerosis in the right vertebral artery. Histologic sections from the aneurysm and the adjacent arteries were stained with hematoxylin and eosin and with Verhoeff-Van Gieson's elastic tissue.

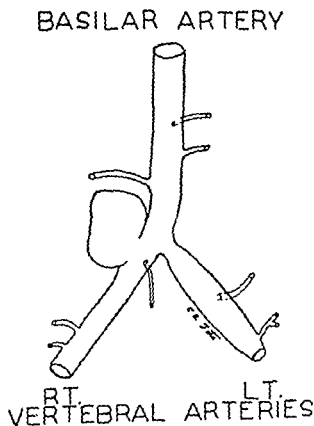


Fig. 1.—A diagrammatic sketch of the aneurysm in Case 1.

The intima, the internal elastic lamella, and the media were continuous from the wall of the right vertebral artery into the adjacent portion of the aneurysm. The intima became thicker, the internal elastic lamella soon completely disappeared, and the media rapidly decreased in the wall of the aneurysm. Only a small amount of adventitia was present in the wall of the sac. Many mononuclear cells infiltrated the tissues about the aneurysm.

The intima of the right vertebral artery was thickened in the area of the aneurysm. There was some atherosclerosis in the vessel, although there was no evidence of an inflammatory reaction. The internal elastic lamella was fragmented and in some areas it was absent in the wall of the right vertebral artery just opposite the orifice of the aneurysm (Fig. 2).

The wall of the basilar artery in the area proximal to the union of the vertebral arteries showed many variations in the proportion of the intima to the media and to the adventitia. At one point the internal elastic lamella was absent and the media was hypertrophied, while in another area the media was completely absent and the wall was formed primarily by intima and only a thin layer of adventitia (Fig. 3).

tance of 1 cm. between the points of union of the vertebrals and the circle of Willis. Histologically there was a variation in the thickness of the different portions of the wall, and in some areas the internal elastic lamella was completely absent (Fig. 4). There were neither macroscopic nor microscopic changes observed in the remaining seven specimens. Macroscopic variations in the size, position, and the relationship of the vertebrals and basilar arteries have been frequently observed; however, it seems that histologic studies have been made upon these vessels very infrequently.

#### SUMMARY

The etiology of the aneurysms occurring on the vertebral arteries is discussed. It is suggested that congenital defects occurring in the wall of these arteries play a significant role in the formation of saccular aneurysms. Aneurysms may develop as a result of other processes; however, vascular defects must be considered even in those individuals who have either syphilis or arteriosclerosis.

A total of seventy cases of aneurysms occurring on the vertebral arteries have been previously reported. Two additional cases are added to the literature. It is obvious, therefore, that aneurysms are infrequent on the vertebral arteries.

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## RESISTANCE OF THE MELBOURNE STRAIN OF INFLUENZA VIRUS TO DESICCATION\*

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THE ability of disease germs to survive in a potent state entirely apart from their living animal hosts is a property quite variable for different kinds of microbes. Some may remain virulent for years, while others seem to become innocuous almost at once. The nature of the environmental medium, temperature, and humidity also play important roles. The persistent potency of a particular pathogenic microbe under various conditions may best be ascertained by actual observation and experimentation. Edward<sup>1</sup> in 1941 reported the recovery of potent influenza virus many hours after drying on cloth, on glazed surfaces, and in household dust; in one experiment the dry virus remained potent for five weeks. This matter would seem to be of sufficient gravity to merit further experimental investigation.

In the present study we have subjected the Melbourne strain of influenza A virus to drying outside the living host and have then introduced this into embryonated eggs to ascertain its ability to infect. The Melbourne virus was maintained by serial passage in embryonated eggs. At least once a week 0.05 c.c. of virus-infected allantoic fluid was injected into the extraembryonic fluids of seven-day embryonated eggs. On the second day after inoculation, the embryo died and its allantoic fluid was harvested to serve as the virus-infected fluid for further propagation of the virus and for the experimental tests. The titer of virus in this fluid was measured by preparation of decimal serial dilutions which were injected into embryonated eggs, and it was found to be quite variable, from  $10^{-1}$  to  $10^{-8}$ . In other words it was sometimes found that 0.1 c.c. of a 1 to 100,000,000 dilution ( $10^{-8}$ ) of the allantoic fluid was sufficient to cause typical death of half the eggs inoculated, while occasionally this failed and only less dilute preparations would cause death; in rare instances as much as 0.1 c.c. of a 1 to 10 dilution of the allantoic fluid was required. At frequent intervals the virus was tested by intranasal administration to white mice in order to check against the possibility that the influenza virus had been lost and accidentally replaced by some other virus lethal for chick embryos. The technical procedure of inoculation, incubation, candling, and eventual autopsy of the eggs has been previously described in publications from this laboratory. Occasional tests for bacterial contaminations were made, and occasional eggs obviously dying from causes other than infection with the virus were excluded from consideration.

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## ANEURYSMS OF THE VERTEBRAL ARTERIES; A CONSIDERATION OF THEIR ETIOLOGY\*

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THE frequency of intracranial aneurysms is indicated by the following observations: Fearnside<sup>1</sup> found 44 cases in a group of 5,432 autopsies; Osler<sup>2</sup> observed 12 in 800 autopsies; Pitt<sup>2</sup> found 19 in approximately 9,000 autopsies; and Conway<sup>3</sup> found 43 cases of undoubted cerebral aneurysms among 6,325 sections. The vessels in the intracranial cavity have more aneurysms upon them than any other group of arteries of corresponding size in the body. The thinness of the wall of these arteries and the absence of supporting tissue may be factors in the frequent development of these lesions.

Bradford,<sup>4</sup> in discussing the frequency of aneurysms on the cerebral vessels, says: "One cannot help thinking sometimes that the incidence of aneurysms of the cerebral vessels must be linked to the difference in the physiology of the cerebral circulation as compared with such a circulation as that of the viscera. The cerebral arteries are engorged passively whenever the tension rises in the systemic vessels . . . they are passively distended as the result of the increased aortic pressure which is transmitted to them through the column of blood."

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Arteriosclerosis according to Dial and Maurer<sup>6</sup> undoubtedly plays the major role in the formation of intracranial aneurysms. Seven of their thirteen cases are considered to be associated with arteriosclerosis. The observation of McDonald and Korb<sup>5</sup> that "in 49.5 per cent of the 572 cases in which the vessels were described there were sclerotic arteries" may indicate the role of arteriosclerosis in the formation of these aneurysms. Keegan and Bennett<sup>7</sup> express the opinion, however, that arteriosclerosis is rarely the cause of cerebral aneurysms. Lebert,<sup>8</sup> as early as 1866, says, "Calcifying atheroma is rarely found in cases of cerebral aneurysm and is not an important cause of their formation." Hoffman<sup>9</sup> in 1894 likewise concluded from a study of 78 cases that atheroma was not a common cause in the formation of aneurysms; however, atheroma and a heightened blood pressure were important factors in the causation of rupture. In a large group of cases of intracranial aneurysms Parker<sup>10</sup> could find no evidence of arteriosclerosis anywhere in the body.

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of which are not given. Seven months before death he "fell out." There is no history relative to this episode.

*Pathologic Examination.*—Only the pertinent findings are recorded. The autopsy was done one hour following death. There was considerable blood in the subarachnoid space, especially over the occipital and parietal lobes. In the area of the medulla the blood was clotted and appeared older than that in the other portions of the brain. A small amount of blood was present in the tip of the lateral ventricles. In serial sections through the brain several small infarcts were found, one of which was a recent lesion. The source of the subarachnoid hemorrhage was an aneurysm located on the lateral portion of the right vertebral artery approximately 2 mm. proximal to the basilar artery (Fig. 1). This aneurysm measures 1.5 cm. in diameter and was partially filled with a thrombus. The wall was thin and at one point it had ruptured. Old blood was present in the tissues around the pons and the medulla.

There was considerable atherosclerosis in the left vertebral artery and in the basilar; however, there was only a minimal amount of sclerosis in the right vertebral artery. Histologic sections from the aneurysm and the adjacent arteries were stained with hematoxylin and eosin and with Verhoeff-Van Gieson's elastic tissue.

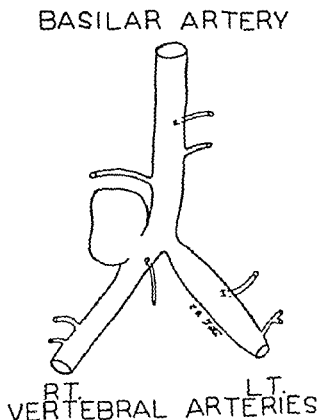


Fig 1.—A diagrammatic sketch of the aneurysm in Case 1.

The intima, the internal elastic lamella, and the media were continuous from the wall of the right vertebral artery into the adjacent portion of the aneurysm. The intima became thicker, the internal elastic lamella soon completely disappeared, and the media rapidly decreased in the wall of the aneurysm. Only a small amount of adventitia was present in the wall of the sac. Many mononuclear cells infiltrated the tissues about the aneurysm.

The intima of the right vertebral artery was thickened in the area of the aneurysm. There was some atherosclerosis in the vessel, although there was no evidence of an inflammatory reaction. The internal elastic lamella was fragmented and in some areas it was absent in the wall of the right vertebral artery just opposite the orifice of the aneurysm (Fig. 2).

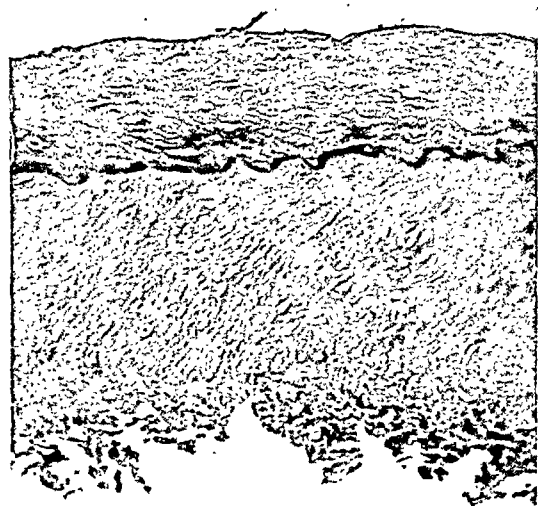
The wall of the basilar artery in the area proximal to the union of the vertebral arteries showed many variations in the proportion of the intima to the media and to the adventitia. At one point the internal elastic lamella was absent and the media was hypertrophied, while in another area the media was completely absent and the wall was formed primarily by intima and only a thin layer of adventitia (Fig. 3).

The prostate was slightly enlarged. The wall of the urinary bladder was hypertrophied. There was some cystitis and pyelonephritis present.

*Anatomic Diagnosis.*—There were multiple defects in wall of right vertebral and basilar arteries. Aneurysm of the right vertebral artery with perforation, subarachnoid hemorrhage, clotted blood in lateral ventricles, bronchopneumonia, generalized arteriosclerosis, infarcts in brain, old and recent, benign prostatic hypertrophy, and acute and chronic cystitis and pyelonephritis were also present.



A.



B.

Fig. 2.—A. The internal elastic lamella is fragmented and in some areas it is absent. This section is from the right vertebral artery opposite the orifice of the aneurysm. Verhoeff-Van Gieson's stain.  $\times 90$ . B. Normal portion of the wall of the right vertebral artery. Verhoeff-Van Gieson's stain.  $\times 90$ .

CASE 2.\*—M. R., a 42-year-old white male, had a sudden attack of weakness in both legs while driving his automobile. A severe pain located in the base of his skull accompanied the

\*This autopsy was done in 1934 while one of us was at Duke University. I wish to thank Dr. W. D. Forbus, Professor of Pathology, Duke University, for permission to use it in this paper.

onset of this weakness. The patient stated that it seemed as if his heels were coming up to meet the back of his head. Symptomatic relief was given for five days, during which time there was an intense, throbbing, occipital headache.

On admission to the hospital, five days following the onset, the neck was stiff and there was a bilateral positive Kernig. The blood pressure was 150/110. The spinal fluid was under an increased pressure and was uniformly bloody. The amount of blood in the spinal fluid decreased during the following three days. At this time he was seized with a sudden violent pain in the head and neck and quickly went into shock. Fresh blood occurred in the spinal fluid. During the following month, there were several occasions in which fresh bleeding occurred into the spinal fluid. The final attack was characterized by a violent convulsion, whereupon the patient went into shock and then died.

The patient had a high fever at intervals during hospitalization. There was persistent mental disturbance. The Wassermann reaction was negative on both the blood and the spinal fluid.



Fig. 3.—Section of the basilar artery showing a variation in the relationship of the intima to the media and adventitia. The internal elastic lamella is absent in some areas. Verhoeff-Van Gieson's stain.  $\times 48$ .

**Pathologic Examination**—The autopsy was done approximately eighteen hours after death. There was a large amount of blood about the base of the brain especially in the area of the pons and the medulla. The blood was fresh in some areas while in others it was old. The source of this hemorrhage was an aneurysm on the left vertebral artery. It measured 1.6 cm. in diameter and was located just opposite the point of union of the two vertebral arteries. The aneurysm was sacular and was filled with a thrombus. At one point in the wall there was a fresh thrombus; apparently this was the site of perforation. The vertebral artery was dilated near its union with the left. The basilar was also dilated and was distorted from its normal position. The histologic sections from the aneurysm and the adjacent blood vessels were not satisfactory for study. The extensive sclerosis made the vessels unsuitable for sectioning. Mononuclear cells with pigment and red blood cells were present in the wall of the aneurysm and in the surrounding tissues. This reaction apparently was secondary to the rupture. The arteries throughout the body showed considerable atherosclerosis. There was no pathologic change in any of the vessels that suggested syphilis. There was a cyst in the left kidney that measured 1.5 cm. in diameter.

**Anatomic Diagnosis**.—Generalized arteriosclerosis, aneurysm of the left vertebral artery with rupture, subarachnoid hemorrhage, obstruction of foramen of Luschka on right and left, hydrocephalus, clotted blood in all ventricles, cyst in left kidney were present.

#### DISCUSSION

The pathologic findings in these cases apparently are typical of those reported by others. In Case 1 the amount of arteriosclerosis is that which one

may find in the cerebral vessels at the age of fifty years. The location of the aneurysm is opposite the point of union of the two vertebrals, and it is not in the angle formed by a branch leaving the vertebral artery. It is suggested that defects in the wall of the right vertebral artery at the site of the aneurysm similar to those in the opposite wall of the vertebral artery and in the basilar artery may have been the bases for the development of this aneurysm. Since syphilis is such a common infection in the Negro in this section of the country, one has to be careful in considering its role in pathologic processes. There is no pathologic evidence of syphilis in Case 1. Since the spinal fluid contained blood, one must be careful in interpreting the Wassermann reaction in this case.

The arteriosclerotic process is most extensive in Case 2. Such a lesion, however, may be found in the cerebral vessels even at 42 years of age. The abnormal size and position of the vertebrals and the basilar artery as observed in this man occur frequently in the cases of aneurysms reported in the literature. Morrow's case<sup>18</sup> is one of a fusiform aneurysm,  $2.5 \times 3.0$  cm., located on the right vertebral artery. The basilar artery is dilated in Wells'<sup>19</sup> case. One of the two cases reported by Bassae'<sup>17</sup> also has multiple aneurysms on the basilar artery. Both vertebral arteries are the site of aneurysms in Ruston and Southland's case.<sup>20</sup> It may be concluded, therefore, that abnormalities of the basilar and vertebral arteries are frequent. Parker<sup>10</sup> says "congenital weakness of the wall of a cerebral artery with the formation of aneurysm may be associated with congenital anomaly elsewhere in the vascular system." We have not observed such a relationship, however, in our cases.

The presence of arteriosclerosis in the vertebral, the basilar, and the cerebral arteries must be considered very carefully before concluding that it is the etiology of the saccular aneurysms that occur on these vessels. Furthermore, it is difficult for us to conceive of only 70 cases of aneurysms having been reported on the vertebral arteries if an atheromatous process is a significant factor in their development. If sclerosis is important in the etiology of saccular aneurysms, similar lesions should occur on other vessels, such as the coronary. Observations on coronary aneurysms have been made recently by one of us.<sup>21</sup>

Statistics show that aneurysms on the vertebral arteries occur more often after 30 years of age. It is significant, however, in considering the etiology of such aneurysms to know that similar lesions have occurred in younger individuals. The case reported by Dial and Maurer<sup>6</sup> occurred in a child two years of age. It is almost identical macroscopically with the two cases reported in this paper. This baby received a fracture of the skull in an automobile accident "with apparent recovery after five days," and two days later there was an onset of convulsions, dyspnea, and cyanosis. Death occurred thirty-six days after the accident, from the rupture of an aneurysm on the right vertebral artery. The authors say "the youth of the patient, 2 years, and the fact that the microscopic appearance of the aneurysm suggests a malformation of the vessels are factors pointing to a congenital lesion. The question as to etiology on a traumatic or congenital basis cannot be decided absolutely." Fearnside's<sup>1</sup> in a large series of intracranial aneurysms could find no relationship between trauma and the development of the aneurysm.

The aneurysm reported by Duguid<sup>22</sup> in a boy 17 years of age, occurring

at the junction of the left vertebral artery with the basilar, is important in considering the etiology of aneurysms. The intimal coat of the vertebral artery was thickened at the site of the aneurysm. The internal elastic lamella was split and the media was absent. The author concluded that "the case seems to illustrate a point which, although not uncommon in aneurysms of the vertebral vessels, is not often noted; namely, that aneurysms may be formed by a pouching of a fibroid intima through a deficient or severed media."

Congenital defects in the wall of the cerebral arteries have been observed by Busse<sup>23</sup> and Emrich.<sup>24</sup> These defects were studied especially in the anterior cerebral arteries and gave rise to the so-called "Dehnungsaneurysmen." Morphologic variations of a gross character are extremely common in the arteries forming the circle of Willis. Minor changes in the elastic layers of the media of the arteries are also common in the arteries forming the circle of Willis. Minor changes in the elastic layers of the media of the arteries are also common in the cerebral arteries of persons who show no gross aneurysmal dilatations.<sup>1</sup>

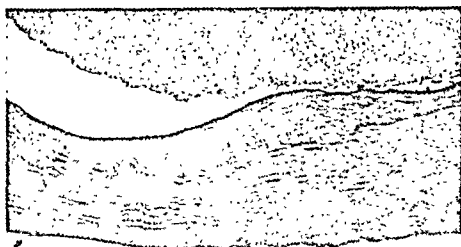


Fig. 4.—The internal elastic lamella is split and it is absent in some portions of the basilar artery. There is a macroscopic malformation in this artery. Verhoeff-Van Gieson's stain.  $\times 96$ .

Intracranial aneurysms are frequently explained on the bases of congenital defects; however, from a review of the literature, such defects are infrequently considered as the etiologic agent in the formation of aneurysm on the vertebral arteries. Of the intracranial aneurysms collected by McDonald and Korb<sup>6</sup> 32.7 per cent are referred to as congenital in origin; however, only three of the 68 aneurysms occurring on the vertebral arteries are considered as congenital. In two of these three cases, however, the authors questioned the possibility of a congenital basis for the aneurysms.

After a review of the seventy cases of aneurysms occurring on the vertebral arteries and the two additional cases reported in this paper, it is suggested that these aneurysms more frequently have their origin in congenital defects that are present in the wall of the arteries.

The vertebral and basilar arteries were studied in a group of eight cases to determine the presence or the absence of malformations and histologic defects in the vessel walls. Six of these were from adults and two from newborn infants. A malformation occurred in only one of these and that was in an adult. In this specimen the basilar artery was divided into two vascular channels for a dis-

tance of 1 cm. between the points of union of the vertebrals and the circle of Willis. Histologically there was a variation in the thickness of the different portions of the wall, and in some areas the internal elastic lamella was completely absent (Fig. 4). There were neither macroscopic nor microscopic changes observed in the remaining seven specimens. Macroscopic variations in the size, position, and the relationship of the vertebrals and basilar arteries have been frequently observed; however, it seems that histologic studies have been made upon these vessels very infrequently.

#### SUMMARY

The etiology of the aneurysms occurring on the vertebral arteries is discussed. It is suggested that congenital defects occurring in the wall of these arteries play a significant role in the formation of saccular aneurysms. Aneurysms may develop as a result of other processes; however, vascular defects must be considered even in those individuals who have either syphilis or arteriosclerosis.

A total of seventy cases of aneurysms occurring on the vertebral arteries have been previously reported. Two additional cases are added to the literature. It is obvious, therefore, that aneurysms are infrequent on the vertebral arteries.

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## RESISTANCE OF THE MELBOURNE STRAIN OF INFLUENZA VIRUS TO DESICCATION\*

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THE ability of disease germs to survive in a potent state entirely apart from their living animal hosts is a property quite variable for different kinds of microbes. Some may remain virulent for years, while others seem to become innocuous almost at once. The nature of the environmental medium, temperature, and humidity also play important roles. The persistent potency of a particular pathogenic microbe under various conditions may best be ascertained by actual observation and experimentation. Edward<sup>1</sup> in 1941 reported the recovery of potent influenza virus many hours after drying on cloth, on glazed surfaces, and in household dust, in one experiment the dry virus remained potent for five weeks. This matter would seem to be of sufficient gravity to merit further experimental investigation.

In the present study we have subjected the Melbourne strain of influenza A virus to drying outside the living host and have then introduced this into embryonated eggs to ascertain its ability to infect. The Melbourne virus was maintained by serial passage in embryonated eggs. At least once a week 0.05 c.c. of virus-infected allantoic fluid was injected into the extraembryonic fluids of seven-day embryonated eggs. On the second day after inoculation, the embryo died and its allantoic fluid was harvested to serve as the virus-infected fluid for further propagation of the virus and for the experimental tests. The titer of virus in this fluid was measured by preparation of decimal serial dilutions which were injected into embryonated eggs, and it was found to be quite variable, from  $10^{-1}$  to  $10^{-8}$ . In other words it was sometimes found that 0.1 c.c. of a 1 to 100,000,000 dilution ( $10^{-8}$ ) of the allantoic fluid was sufficient to cause typical death of half the eggs inoculated, while occasionally this failed and only less dilute preparations would cause death; in rare instances as much as 0.1 c.c. of a 1 to 10 dilution of the allantoic fluid was required. At frequent intervals the virus was tested by intranasal administration to white mice in order to check against the possibility that the influenza virus had been lost and accidentally replaced by some other virus lethal for chick embryos. The technical procedure of inoculation, incubation, candling, and eventual autopsy of the eggs has been previously described in publications from this laboratory. Occasional tests for bacterial contaminations were made, and occasional eggs obviously dying from causes other than infection with the virus were excluded from consideration.

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For most of the drying tests the allantoic fluid containing the virus was placed in sterile test tubes 150 × 25 mm. fitted with perforated rubber stoppers and glass inlet and outlet tubes for passage of the dry air. The air of the room was passed through two glass tubes about 50 cm. in length, filled with granular anhydrous calcium chloride to remove moisture, and then through long glass tubes packed with sterile cotton into the virus tubes in series, and thence through a safety wash bottle to the suction pump. The virus tubes were turned every few minutes to distribute the liquid over their surfaces and favor the drying. The technique was varied somewhat. As a rule the volume of allantoic fluid in each tube was 0.5 or 1.0 c.c., and ordinarily four short glass rods were included in the tube to provide greater surface. In each instance the drying time was recorded. A portion of each specimen was kept in the fluid state for subsequent comparison. Decimal dilutions were inoculated into embryonated eggs, two to four eggs for each dilution, and the greatest dilution causing death of half or more of the embryos was taken as the index of titer. Sterile double-distilled water was used to resuspend the dried virus and for the dilution fluid. Some of the dried specimens were kept for later examination by storage of the original tubes in a dark closet at room temperature, and these specimens were examined after various time intervals.

In one series of experiments the virus fluid was mixed with mucin. Armour's gastric mucin, 6 grams, was mixed with 50 c.c. of 95 per cent alcohol in a sterile, stoppered 250 c.c. flask and allowed to stand at 56° C. for four days. The excess alcohol was then decanted, the flask plugged with sterile cotton, and the remaining alcohol was allowed to evaporate at 56° C. The dry mucin was then suspended by adding sterile water in successive portions of 10 c.c., with continued stirring, to a total amount of 80 c.c. The resulting mixture, containing approximately 7.5 per cent mucin, was stored in the refrigerator. For use in the experiments it was further diluted ten times with water to make 0.75 per cent mucin, and one part of this was added to two parts of the virus fluid so that the final concentration of mucin in the material to be dried was approximately 0.25 per cent. The mucin itself injected into eggs was found to be without lethal effect.

In another series of experiments powdered talc, previously washed with acid, was mixed with the virus fluid before drying. The talc was shown to be without lethal effect when injected into eggs in the quantities employed.

Description of some of the individual experiments may serve to illustrate the variations in technical procedure and the character of the results.

*Experiment of August 28, 1942.*—Undiluted allantoic virus, 1 c.c., was sprayed onto 10 c.c. of powdered talc with vigorous agitation. Then, at intervals, 0.1 c.c. of the mixture was removed, mixed with 1 c.c. sterile water, and injected into several eggs in a dose of 0.1 c.c. It will be noted that the original virus fluid, which was found to have a titer of  $10^{-4}$ , had been subjected to a dilution of 1 in 100 in addition to the drying effect of the talc, so that 1 c.c. of this dilution would still have contained a possible 100 lethal doses and 0.1 c.c. only 10 lethal doses. The test after five minutes' exposure to the talc resulted in five deaths and no survivals; after thirty minutes and again at ninety minutes, at which time the talc powder appeared quite dry, inoculation resulted in death of

all the eggs. After three hours the sample gave three deaths and two survivals, and after ninety-six hours another sample gave one death and four survivals. In this experiment the first evident loss of potency occurred after the mixture appeared dry (three hours), and activity was almost completely lost at the end of four days.

*Experiment of October 11, 1912.*—The undiluted virus fluid alone, in 1 c.c. amounts, was dried by air current in each of several glass tubes, requiring 60 to 75 minutes to dry. After various intervals, the dry material was suspended in water and injected into eggs. The test at two hours resulted in death of all five eggs. Tests after twenty hours and after forty-eight hours allowed all the eggs to survive. The titer of the original virus was above  $10^{-4}$ .

*Experiment of November 25, 1912.*—The virus fluid, 5 c.c., was mixed with 2.5 c.c. of 0.75 per cent watery mucin, distributed in 1 c.c. amounts in glass tubes, and dried by air current at room temperature in eighty to 144 minutes. After intervals of twenty-five, thirty, and seventy hours, five, six, seven, and eight days, the dry material was taken up in 0.8 c.c. of water and injected into five eggs in the usual way. One egg of the five-day lot survived. All other eggs died. The titration of a sample five days after drying showed a titer of  $10^{-3}$ . The titer of the original virus was  $10^{-7}$ . In this experiment the virus evidently retained its potency for the entire period of eight days.

*Experiment of December 9, 1912.*—Virus fluid alone in amounts of 0.5 c.c. in glass tubes was dried by air current at room temperature in thirty-five to ninety minutes. After various intervals the dried material was suspended in 1 c.c. water and injected into eggs. The tests were done at thirty hours, two, four, and seven days. One egg out of five used for the seven-day sample survived. All the others succumbed. The original virus had a titer of  $10^{-8}$ .

On this same day this same virus fluid, 2 c.c., was mixed with 1 c.c. of 0.75 per cent watery mucin, and the mixture was distributed in amounts of 0.75 c.c. in glass tubes and dried by air current in from sixty-five to 105 minutes. The tests by inoculation of eggs were done by resuspending in 1 c.c. water and injecting after the dry material had been allowed to stand for thirty hours, seven, nine, and twelve days. All the eggs died. The dry material after twelve days was found to have a titer of  $10^{-3}$ .

*Experiment of January 19, 1913.*—Virus fluid 0.5 c.c. was dried in each glass tube by air current in ninety to 160 minutes. Subsequent tests were made in the usual way by adding 1 c.c. water and injecting 0.1 c.c. of the resulting suspension into each of five eggs. The test at fifteen days resulted in two deaths, two survivals, and one discarded as unsatisfactory; at twenty-two days, one death and four survivals; at forty-five days, three deaths and two survivals. The original virus was found to have a titer of  $10^{-4}$ .

On this same date, January 19, the same virus fluid, 3 c.c., was mixed with 1.5 c.c. of 0.75 per cent watery mucin, and this mixture was distributed in 0.75 c.c. amounts in glass tubes and dried in air current at room temperature in seventy to ninety-five minutes. The dried material was tested in the usual way after fifteen, twenty-two, and forty-five days, and all the inoculated eggs died. Titration of the specimen at fifteen days showed a titer of  $10^{-3}$ , and again at twenty-two days a titer of  $10^{-1}$ . At forty-five days titration was again carried

out, but only the eggs inoculated with the undiluted first suspension died. The titer of the original, undried virus-mucin mixture, 3 parts, plus water 1 part was found to be  $10^{-4}$ . In this experiment the mucin seemed to afford definite protection to the virus when subjected to drying, so that it remained potent for 45 days even though the original titer,  $10^{-4}$ , was not particularly high.

TABLE I  
SUMMARY OF MORE SIGNIFICANT FEATURES OF THE EXPERIMENTS

EXPERIMENT NO.	DATE	ORIGINAL TITER	DRIED WITH	ACTIVE VIRUS PRESENT
1	Aug. 22	$10^{-3}$	Talc powder	None found at 30 minutes
2	Aug. 28	$10^{-4}$	Talc powder	+ at 3 hours; 0 at 96 hours
3	Sept. 11	$10^{-1}$	Mucin in air	None found at 17½ hours
4	Sept. 18	$10^{-4}$	Mucin in air	+ at 3 hours; 0 at 15 hours
5	Sept. 24	$10^{-4}$	Air current	None found at 3 hours
6	Oct. 2	$10^{-3}$	Air current	+ at 2, 24 hours; 0 at 72 hours
7	Oct. 14	$10^{-4}$	Air current	+ at 2 hours; 0 at 20 hours
8	Oct. 22	$10^{-3}$	Air current	0 at 2 hours
9	Oct. 30	$10^{-7}$	Air current	+ at 2, 4, 6½, and 21 hours
10	Nov. 6	$10^{-6}$	Air current	+ at 4, 6, 24, and 26 hours
11	Nov. 17	$10^{-6}$	Air current	+ at 48, 54, and 80 hours
12	Nov. 25	$10^{-7}$	Mucin in air	+ at 5, 6, 7, and 8 days*
13	Dec. 1	$10^{-7}$	Air current	+ at 51, 74, and 96 hours
14	Dec. 9	$10^{-8}$	Air current	+ at 2, 4, and 7 days
15	Dec. 19	$10^{-8}$	Mucin in air	+ at 7, 9, and 12 days†
16	Dec. 22	$10^{-6}$	Air current	+ at 11 and 15 days; 0 at 22 days
17	Dec. 29	$10^{-6}$	Air current	+ at 8 days; 0 at 22 days
18	Dec. 29	$10^{-6}$	Talc in air	+ at 4 days; 0 at 22 days
19	Jan. 5	$10^{-5}$	Air current	+ at 1 and 15 days; 0 at 22 days‡
20	Jan. 5	$10^{-5}$	Mucin in air	+ at 15, 22, and 29 days; 0 at 50 days
21	Jan. 12	$10^{-4}$	Air jet	0 at 15 days
22	Jan. 19	$10^{-4}$	Air current	0 at 15 days
23	Jan. 19	$10^{-4}$	Mucin in air	+ at 15, 22, and 45 days§
24	Jan. 26	$10^{-3}$	Air current	0 at 22 and 38 days
25	Feb. 10	$10^{-4}$	Air current	+ at once; 0 at 16 days
26	Feb. 10	$10^{-2}$	Air current	0 at 7 days
27	Feb. 17	$10^{-4}$ ¶	Air current	0 at 9 and at 15 days
28	Feb. 24	$10^{-3}$	Air current	+ at once; 0 at 16 and 29 days
29	Mar. 3	$10^{-4}$	Mucin in air	+ at once; 0 at 19 and 23 days
30	Mar. 18	$10^{-4}$	Air current	+ at once; 0 at 8 days
31	Mar. 31	$10^{-2}$	Air current	+ at once and 24 hours; ± at 3 days

\*Dry material tested at 5 days gave a titer of  $10^{-3}$ .

†Dry material tested at 12 days gave a titer of  $10^{-3}$ .

‡Original virus caused typical pneumonia in four mice.

§Dry material tested at 15 days gave a titer of  $10^{-2}$  and at 22 days a titer of  $10^{-1}$ .

||This virus on February 10 was subjected to preliminary dilution 1:100.

¶This virus on February 17 was subjected to preliminary dilution 1:10.

Many more experiments of a similar kind were performed. Some few of them miscarried because of too low potency of the original virus fluid or because of bacterial contamination, and some resulted only in duplication. The 31 significant experiments are listed in summary form in Table I, so that the general results may be seen on a single page. The data indicate a considerable range of variation. In some experiments the virus after drying was no longer able to cause death of the embryonated eggs, and in other experiments it was possible to demonstrate lethal virus in the dry material after rather long periods.

Each experiment was carried out without advance information in regard to the titer of the virus being used. In this respect there was a range from  $10^{-1}$  to  $10^{-8}$ . From October 30, 1941, to January 5, 1942, this virus maintained a rather high titer, and the drying tests made during this period revealed the presence of potent virus, for a time at least, in the dry material of every experiment.

#### DISCUSSION OF EXPERIMENTAL RESULTS

Apparently admixture of powdered tale did not favor preservation of the active virus. In Experiment 1 virus could not be recovered after a half hour; in Experiment 2 it was still active at three hours but not at ninety-six hours, and in Experiment 18 it was still active at four days but not at twenty-two days. It is evident that virus of original high titer remained demonstrably active for a longer period. Admixture with powdered tale provides conditions resembling to some extent those present when moist particles from the mouth or nose may fall upon dry dust of the air, floor coverings, or furniture. In such circumstances highly potent virus would seem likely to remain active for four days.

Drying of the allantoic virus on clean glass gave results only slightly different from those obtained with the powdered talc. In some instances the activity of the virus persisted for considerable periods: in Experiment 11 for eighty hours; in Experiment 13 for ninety-six hours; in Experiment 14 for seven days; in Experiment 17 for eight days, in Experiment 16 for fifteen days. In other instances the activity was no longer evident after brief periods: In Experiment 8 at two hours, and in Experiment 5 at three hours. Again the significance of an original high titer,  $10^{-5}$  to  $10^{-8}$ , was evident, and this seemed to make it possible for the virus to retain its activity when dried in an air current even after several (at least fifteen) days. The experimental conditions here resemble those provided when virus expelled from the body comes to rest on a clean glazed surface exposed to moving air, and also to some extent the conditions under which suspended particles dry while suspended in the atmosphere. Virus of high titer may be expected to retain potency longer.

Drying the allantoic virus after admixture of mucin resulted in a rather disturbing revelation of the persistence of virus activity in such material. In particular a virus of high potency tended to retain its activity. In Experiment 12 it was still lethal to eggs after eight days, and at five days the titer was found to be  $10^{-3}$  as compared with an original titer of  $10^{-7}$ . In Experiment 15 even after twelve days the titer was found to be  $10^{-3}$  as compared with an original titer of  $10^{-8}$ . In Experiment 20 the lethal potency for eggs was still present at the end of twenty-nine days in the dry state and in Experiment 23 after forty-five days. The conditions in these experiments mimic those which naturally exist when larger particles of mucus impregnated with virus of high potency are deposited on glazed surfaces, and conditions are probably not significantly different when such mucus is deposited on fabrics such as gauze mask or kerchief. Evidently in the presence of virus of high titer, which we may assume to exist during a severe *pandemic*, it will be unwise to neglect the menace of possibly infected dust deposits in rooms and dust from dried mucus on fabrics or utensils. Even in the face of the less malignant and more common *epidemic*

influenza, the possible danger of transmission *per fomites* may well receive attention.

#### CONCLUSIONS

1. Experimental tests of the effect of ordinary desiccation by drying in an air current indicate that the Melbourne strain of Influenza Virus A of *low potency* is inactive a short time after drying, or at least impaired to such a degree that the virus no longer brings about death of embryonated eggs inoculated by a standard technique.

2. When, however, the original virus is of *high potency*, such as may be assumed in *pandemic* disease, then this is no longer true. Such virus remains lethal to eggs for many days after drying in air.

3. Highly potent virus mixed with mucus and dried in the air may retain its lethal potency for embryonated eggs for as long as forty-five days.

4. The transmission of epidemic and pandemic influenza through the agency of dust and dry fomites is a distinct possibility, not to be ignored in the practical control of these diseases.

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## THE APPARENT ADVANTAGE OF FREQUENTLY ADMINISTERED QUININE IN AVIAN MALARIA INFECTIONS\*

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IN A RATHER close study of the literature of clinical and experimental malaria over many years the senior author has never encountered the record of an attempt to effect early arrest of the disease by the application of continuous specific therapy from the moment of diagnosis. In 1940, Most and Jolliffe<sup>1</sup> reported the giving of 10 grains (0.6 Gm.) of quinine dihydrochloride intravenously in 10 c.c. of physiologic saline solution every three to four hours for twenty-four hours in the presence of cerebral involvement in patients infected with *Plasmodium falciparum*; but this is intensive rather than continuous therapy and is not likely to win general approval in view of the feeling that such therapy may precipitate blackwater fever. In 1932, St. John<sup>2</sup> gave 10 grains (0.6 Gm.) of quinine sulfate every four hours for four or five days to eight patients and found that high concentrations of quinine could be maintained in the blood by this method, but the effects upon the parasitological and clinical course of the disease could not well be studied by him since his patients were only afflicted with the quickly responding induced malaria. The Fourth General Report of the Malaria Commission of the League of Nations Health Organization<sup>3</sup> discusses the experimental treatment and prophylaxis of malaria in 12,288 subjects, but deals with drugs only in terms of total daily dosages. Of course, the almost universal custom throughout the world is to give the first dose of either quinine or atabrine as soon as the diagnosis is made and thereafter to give one-third the total daily dosage after each of the three meals; this is the method practically routinely employed in the Allied armed forces. Strong, in his new authoritative sixth edition of Stitt's "Tropical Diseases,"<sup>4</sup> advocates the employment of three daily doses, but he goes a bit further and says that "in certain very severe infections it may be advisable to give even four doses . . . during the first twenty-four hours of the attack." Manson-Bahr,<sup>5</sup> the editor of the British counterpart of Stitt's classical American work, in 1940 stated his feeling that there appears to be an advantage in breaking up the daily dosage into six portions, but in more recent publications he has said nothing about a dosage of this sort. In view, therefore, of the lack of evidence that frequent, to say nothing of continuous, therapy with quinine had ever been given a trial in acute malaria infections in the human being we have sought to investigate the possible advantages of such a method of treatment by putting the matter to experimental trial in avian infections in the laboratory. Two facts seemed further to make

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this investigation worth while: first, that quinine is rapidly destroyed and excreted by the body; and, second, that in bacillary infections the frequent administration of sulfonamides is a *sine qua non* of success.

#### METHOD

In all the malaria work in our laboratory in recent years we have used the female canary as host animal and infected her by the direct bite of *Culex pipiens* mosquitoes carrying the very virulent strain of *Plasmodium cathemerium* 3H2<sup>6</sup> developed by us through about six years of uninterrupted canary-mosquito-canary passage. In the beginning of the present study our aim was to set up three groups of infected birds, one to be used as untreated controls, one to be given one-third the total quinine dosage in three equal portions through twelve hours of each day, and one to be given the same total daily dosage but administered through continuous drip during the twelve hours. However, we failed utterly in attempts to devise a satisfactory method of continuous administration to the canary. The intravenous route was of course out of the question and even the intraperitoneal or intragastric drip was made impossible by the size and the squirming and fluttering propensities of the bird. In the end we had to give up the hope of effecting twelve hours of continuous administration and resort to 12 doses during twenty-four hours in the one group, 3 doses in twelve hours in the other group, and no treatment at all in the control group. This gave us a comparison of three times daily dosage and very frequent (i.e., every two hours) dosage, but it did not enable us precisely to study the effect of "continuous" administration as originally contemplated.

Thirty-six birds were bitten for the study, but only the 30 in whose blood we found plasmodia late on the night of the fourth day were used. On the morning of the fifth day (Monday) these 30 infected birds were divided into 3 lots of 10 each and smears of the peripheral blood were made, beginning the smearing at 7:30; smears were thereafter made daily at this time through Friday. The dosing proceeded as follows: *drug*: quinine bisulfate in aqueous solution prepared freshly each day; *dosage*: the total dosage administered to both treated groups in each twenty-four hours was 3 mg., the group receiving the 3 large daily doses being given 1 mg. in 0.1 c.c. of water at 8 A.M., 2 P.M., and 8 P.M., and the group receiving the 12 small doses being given 0.25 mg. in 0.05 c.c. of water at 8 A.M., 10 A.M., 12 N., 2 P.M., 4 P.M., 6 P.M., 8 P.M., 10 P.M., 12 M., 2 A.M., 4 A.M., 6 A.M., etc. (the controls were given 0.05 c.c. of water at these same two-hour intervals); *route of administration*: oral, dribbling the solution into the mouth through a blunted 27 gauge needle; *length of treatment*: the first four days of the infections, Monday through Thursday, inclusive. Thus there were 10 birds dosed three times daily for four days, 10 birds given suitably reduced doses every two hours round the clock continuously for four days, and 10 birds held as untreated controls.

The relative effectiveness of the two types of treatment was determined by (1) the respective heights of the daily plasmodial counts in all groups, employing the counting technique which we have recently described;<sup>7</sup> and (2) the relative retardations of the rates of development of the plasmodia, employing the same

criteria of retardation as have been previously used in this laboratory.<sup>8</sup> The findings are presented in Chart I and Table I.

#### ANALYSIS OF FINDINGS

Upon studying Chart I it is seen that in the *control* group, 1 bird reached crisis (i.e., the numerical peak in the daily plasmodial counts) on the third day, and 9 reached crisis or a near plateau on the fourth day. In the group dosed 3 times daily, 3 birds reached crisis on the third day, 5 reached crisis or a near plateau on the fourth day, and 2 continued to rise sharply after the fourth day. In the group dosed every two hours, 4 birds reached crisis or a near plateau on the second day, and all the birds had reached or passed crisis by the third day. To recapitulate: in the control group, 9 of the 10 birds did not reach crisis or plateau until the fourth day; in the group dosed 3 times daily, 8 of the birds reached this point on the third or fourth day; and in the group dosed every two hours, all birds had reached this point on the second or third day. The plotted average course of the infections in the three groups reveals several points very sharply: (a) Upon the third day, the count was about the same in the two treated groups, but this count marked the peak of the rise in the group dosed every two hours, whereas the peak was not reached until one day later (and then at a considerably higher level) in the other group; (b) upon the fourth day, the count in the group dosed every two hours was only 55 per cent as high as in the other group; (c) upon the fifth day, the count in the group dosed every two hours was only 33 per cent as high as in the other group, and it is further notable that the count in the latter group was still considerably higher than it had been even at the peak in the more frequently dosed group.

TABLE I  
PERCENTAGES OF TROPHOZOITES OF SIZES INCREASING FROM A TO D  
(AVERAGES FOR ALL MEMBERS OF EACH GROUP ON THE "PEAK" DAY)

BIRD GROUP	A	B	C	D	SMALL (A + B)	LARGE (C + D)
Controls	9.6	39.2	38.9	21.2	39.8	60.1
3 doses	23.7	42.3	15.1	8.9	76.0	24.0
12 doses	88.0	10.9	0.3	0.8	98.9	1.1

For an understanding of Table I it is necessary to recall that among the morphologic evidences of specific chemotherapeutic effect upon the malarial plasmodium perhaps most marked is the retardation of its rate of growth. In terms of the present study this effect is recorded in Table I, compiled from data obtained as follows: Each smear which represented the peak day of its respective infection was re-examined and the relative sizes of the organisms in 100 singly infected erythrocytes were noted; the numbers of organisms of sizes A, B, C, and D were then separately added for each of the three groups of birds, averages were determined, and these averages were recorded in terms of percentages of the respective sizes to form the basis for Table I. The data seem clearly to indicate that frequent proportionately reduced quinine dosage is able to effect a greater retardation in trophozoite development than three times daily dosage, since with the more frequent dosage only 1.1 per cent of the organisms had attained a large size at smearing time while 24.0 per cent had done so in the less frequently dosed group.



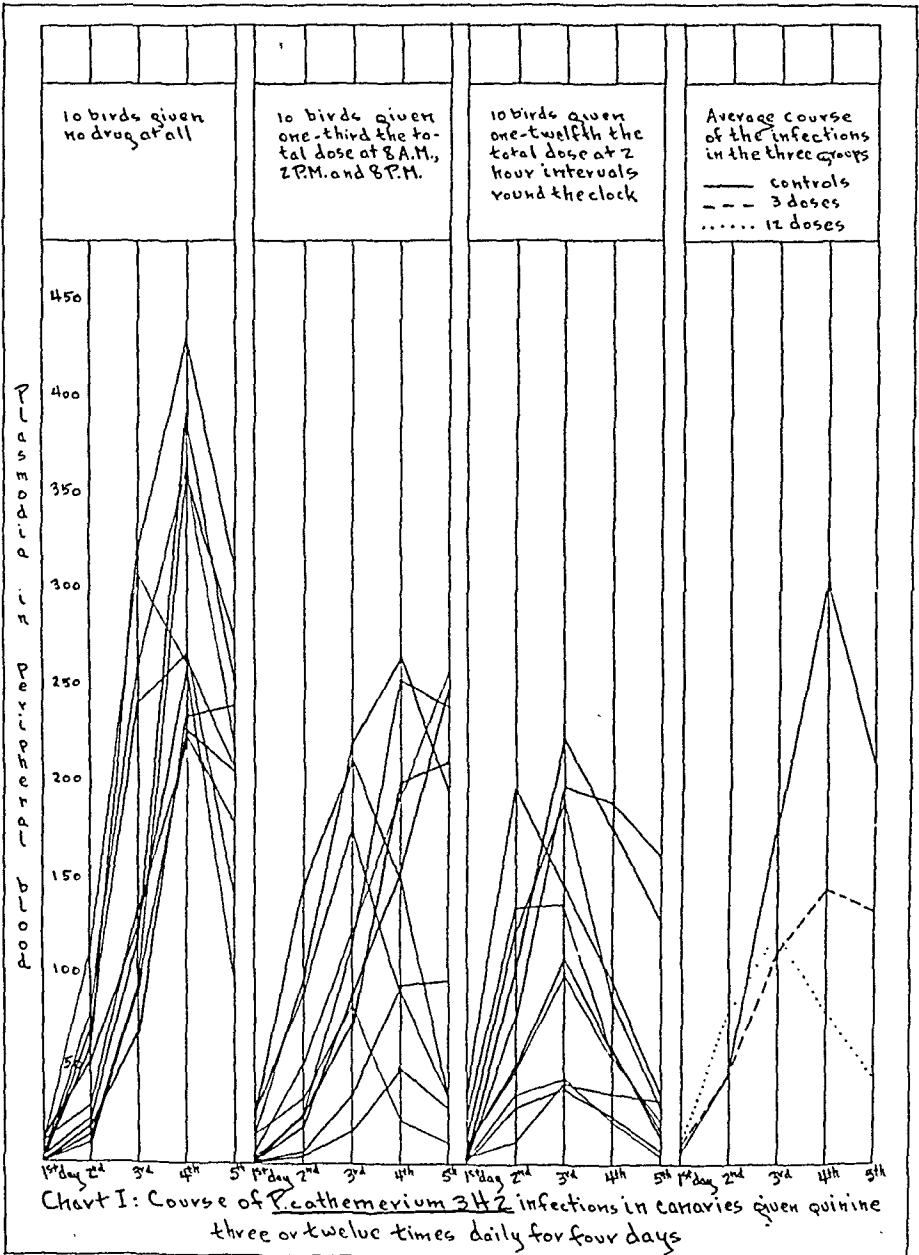


Chart I.

# CLINICAL CHEMISTRY

## A STUDY OF THE EFFECTS OF VITAMIN D ON CAPILLARY PERMEABILITY BY THE USE OF THE DYE T-1824\*

A. SILVER, M.S., IRVING E. STECK, M.D.,† AND C. I. REED, PH.D., CHICAGO, ILL.

THE influence of calcions in decreasing membrane permeability has been recognized for a long time. Some observations on the reduction of edema in arthritic joints suggested the possibility of a causal relationship between this reaction and the calcium-mobilizing action of massive doses of vitamin D. It is the purpose of this paper to make a progress report on the results of some experiments on dogs designed to investigate this possibility.

The method used for determining changes in capillary permeability was the disappearance rate of the dye T-1824 from the blood stream. This method has been suggested as applicable in shock by Price and Longmire.<sup>1</sup> Tislowitz<sup>2</sup> reported on the use of the "endurance-time" of Congo red in the blood stream as an indicator of capillary permeability.

### METHOD

Throughout this work, adult male dogs of 14 to 20 kilograms body weight were used. In determining the disappearance rate of the dye from the blood stream, the technique was similar to that used in routine plasma volume determinations, except that in most cases only two dyed blood samples (10 c.c. each) were withdrawn at fifteen and sixty minutes respectively after the dye injection.

Hemolysis was minimized by using oiled syringes and tubes and by centrifuging samples before clotting occurred. Later, the serum was expressed from the clotted plasma by pressure. The concentration of dye in the serum samples was determined by a photometer as recommended by Gibson and Evelyn.<sup>3</sup> The per cent disappearance rate in a standard interval was calculated as the percentage difference in concentration of dye between the first and second samples. Plasma volumes were calculated to the nearest 10 c.c. from the concentration of dye in the 15-minute sample.

Eleven trained dogs were used for the chronic work, each animal serving as its own control. After suitable rest periods, five of these were subjected to one or more repetitions of the general procedure. Additional dogs were used for the acute lymph experiments.

Vitamin D was administered orally in capsules, each of 50,000 international units of antirachitic potency.

\*From the Department of Physiology, University of Illinois.

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## DISCUSSION

Failing in our attempt to devise a satisfactory method of continuously administering quinine to canaries we were obliged to fall back upon very frequent dosing, but even this compromise arrangement yielded a startlingly affirmative answer to the question we had asked ourselves: namely, may a given total dose of quinine, administered during a fixed period of time, be more effectively employed by continuous infusion than in the traditional three times daily manner? Specifically, in our "continuously" treated birds the plasmodial counts were lower, the break in the upward trend of the counts came on the second instead of on the third day, the crisis came on the third instead of on the fourth day, the peak at crisis was lower, the counts at the end of the period of observation were only slightly higher than they were at the beginning, whereas in the traditionally treated group they were still higher than they had been even at the peak in the continuously treated group; furthermore, the continuous type of treatment effected an approximately 20 times greater retardation in trophozoite development than did the traditional type of treatment. Our strain of avian malaria being notoriously resistant to drugs, we believe these findings would amply justify comparable studies in the human being. The senior author therefore suggests that the following therapeutic trials might be worth making where large numbers of hospitalized malaria patients are being seen: (a) continuous venoclysis with physiologic saline solution so fortified with a quinine salt that the patient shall obtain 30 grains (2.0 Gm.) of the drug in each 24-hour period; (b) the same as the preceding but with a reduced amount of quinine; (c) the venoclysis maintained for only twelve hours of each day with the quinine content doubled; (d) any of the above plus a small dose of quinine by mouth or intramuscularly at the beginning; (e) the same as any of the above except that the saline-quinine solution be administered by intragastric drip as in the aluminum hydroxide treatment of peptic ulcer.<sup>9</sup>

## CONCLUSIONS

In canaries infected with a very virulent strain of malarial plasmodium, the "continuous" is markedly superior to the "traditional" type of quinine therapy. Trial of such "continuous" therapy in man seems indicated.

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# CLINICAL CHEMISTRY

## A STUDY OF THE EFFECTS OF VITAMIN D ON CAPILLARY PERMEABILITY BY THE USE OF THE DYE T-1824

A. SILVER, M.S., IRVING E. STECK, M.D.,† AND C. I. REED, \*

THE influence of calcions in decreasing metabolism has been recognized for a long time. Some observations on arthritic joints suggested the possibility of a reaction and the calcium-mobilizing action of vitamin D. The purpose of this paper is to make a program of experiments on dogs designed to investigate this problem.

The method used for determining changes in the disappearance rate of the dye T-1824 from the blood has been suggested as applicable in shock by P. A. Levine, reported on the use of the "endurance-time" of the dye as an indicator of capillary permeability.

### METHOD

Throughout this work, adult male dogs were used. In determining the disappearance rate of the dye from the stream, the technique was similar to that used in previous experiments, except that in most cases only two samples were withdrawn at fifteen and sixty minutes after injection.

Hemolysis was minimized by using oiled centrifuge tubes and centrifuging samples before clotting occurred. The dye was removed from the clotted plasma by pressure. The concentration of the samples was determined by a photometer (Evelyn).<sup>3</sup> The per cent disappearance rate in the blood was calculated as the percentage difference in concentration of the dye in the two samples. Plasma volumes were calculated to determine the concentration of dye in the 15-minute sample.

Eleven trained dogs were used for the control experiments. Each dog was used as its own control. After suitable rest periods, the dye was injected and one or more repetitions of the general procedure were followed in the acute lymph experiments.

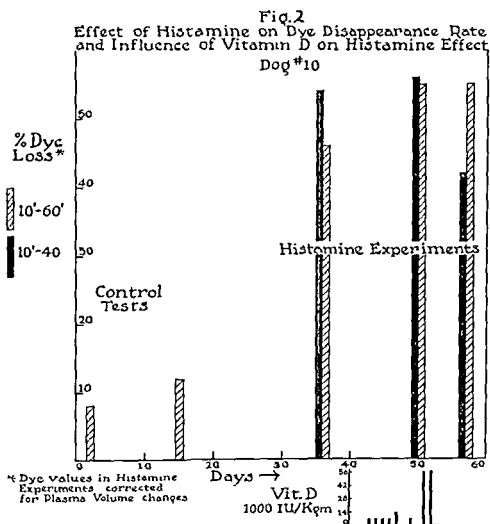
Vitamin D was administered orally in capsules of known units of antirachitic potency.

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minutes' data in evaluating the effect of vitamin D on dye loss, because after 60 minutes, in most cases, the dye level depended on lymph return of dyed fluid to a far greater extent than at 40 minutes, the latter thus giving a truer picture of rate of dye loss. After the administration of vitamin D, the percentage of dye lost in 40 minutes was significantly less than the values before vitamin D, in the case of both animals.

*III. Relation Between Appearance of T-1824 in the Lymph and Loss From the Blood Stream.*—The purpose of these experiments was to find whether dye injected intravenously could be recovered in the lymph in amounts bearing any constant relationship to the amounts disappearing from the blood stream. Ferrebee, Leigh, and Berliner<sup>4</sup> reported on the same procedure, but gave quantitative data in only one case.



Eight dogs were anesthetized with sodium barbital (0.25 Gm./kg.), and their thoracic or cervical lymph ducts were exposed and cannulated. Lymph was collected during a control period, and at two (at least) 15-minute periods after the intravenous injection of T-1824, the average time of lymph collection for these samples was 15 and 60 minutes after dye injection. Routine blood samples were taken simultaneously.

In three of the experiments (illustrated by Fig. 3) 0.5 mg./kg. of histamine was injected at approximately the twentieth minute after dye injection, and the blood and lymph specimens were collected as above.

Fig. 4 shows that a linear relationship exists between the change in dye concentration in the blood between the fifteenth and sixtieth minutes after

volume. Vitamin D in large amounts may be one of the latter agents which "contract" the liver. This action would explain the decreased dye disappearance rates, the increased total blood volume, and the antagonism of the histamine effect.

#### SUMMARY

1. A method has been presented for detecting changes in capillary permeability by the determination of the disappearance rate of T-1824 from the blood stream.

2. The disappearance rate of the dye after histamine injection was found to be increased greatly over normal disappearance rates.

3. The increasing concentration of the dye in the lymph after intravenous injection was found to be directly proportional to the decreasing concentration in the blood stream in both normal and histamine-treated dogs.

4. After a latent period of five to eight days following a large dose of vitamin D, these significant changes were found: (a) increased total plasma volume, (b) increased volume per cent of red cells, (c) decrease in dye disappearance rate in normal dogs, (d) decrease in dye disappearance rate in dogs given histamine, and (e) loss of weight which persisted for several weeks.

5. Possible mechanisms of the changes in paragraph 4 are suggested.

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## EXTREME RESISTANCE TO INSULIN STUPOR IN A SCHIZOPHRENIC PATIENT\*

T. D. RIVERS, M.D., AND K. A. C. ELLIOTT, PH.D., PHILADELPHIA, PA.

THE variability in the response of different subjects to similar doses of insulin is observed by all who have experience in the shock treatment of psychotic patients or in the treatment of diabetes. Similarly the effects of low levels of blood sugar are known to vary widely in different subjects. Petersen and Lutz<sup>1</sup> report the case of a patient who failed to go into stupor despite the fact that the blood sugar fell to 15 mg. per 100 c.c. on a dose of 360 units of insulin administered intravenously. We wish to report observations on a patient who shows extreme resistance to insulin stupor.

The routine procedure developed by one of us (T. D. R.) for the insulin shock treatment of psychotic patients is as follows. For a preliminary period of one week, patients receive daily intramuscular injections of 5 units of insulin in order to desensitize those who might show sensitivity to insulin. Then the patient receives, before breakfast, daily doses of insulin which start at 50 units and are increased, usually by 20 units at a time, until stupor is produced.

The subject of this report, a hebephrenic schizophrenic male, 23 years old, had his dosage of insulin increased up to 1,200 units intramuscularly, the later increments in dosage being 100 units at a time. This failed to produce stupor, and insulin was then administered intravenously starting with 20 units and increasing the daily dose by fairly large increments up to 2,500 units. Deep stupor was never produced. After the highest dose, some twitching occurred, but the patient could still drink glucose solution offered at the end of the treatment period. Finally, the patient was given 120 units of protamine zinc insulin intramuscularly at 8 P.M. and then received 500 units of ordinary insulin intravenously at 8 A.M. the next morning. This time, light stupor for about fifteen minutes was produced two and a half hours after the intravenous injection, but a convulsion occurred which awakened the patient. (Brief muscular activity, such as a convulsion, is known to cause a rise in blood sugar. See, e.g., Elliott et al.<sup>2</sup>)

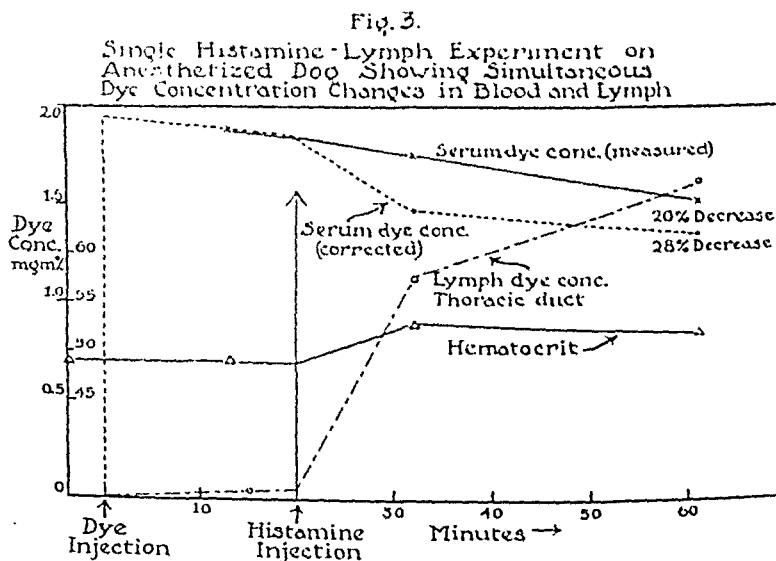
Venous blood sugar levels were determined immediately before and at intervals after the administration of insulin, by the method of Benedict as modified by Elliott et al.<sup>2</sup> Results are shown in Table I. It will be seen that, even with relatively small doses of insulin, the blood sugar fell rapidly, following a course very similar to that observed with ordinary patients who go into deep stupor. Actually, increasing the intramuscular dose from 400 U. (the lowest at which blood sugar determinations were made) to 1,200 U., or the in-

\*From the Institute of the Pennsylvania Hospital.  
Received for publication, October 20, 1943.

dye injection and the simultaneous change in concentration of dye in the lymph. Each point represents one experiment.

#### DISCUSSION OF METHOD

In our opinion, based on the lymph studies and dye disappearance rates after histamine, the per cent disappearance rate of the dye for the hour period after injection is a direct indication of the rate of fluid circulation between the blood and lymph. Normally a certain amount of plasma, with plasma proteins, passes per unit time through capillaries to the tissues and then into the lymphatics. At the same time a similar volume of lymph is returning to the blood stream. The dye T-1824, attached to plasma albumins,<sup>6</sup> is incorporated in this circuit (later removed by phagocytosis), and since normally very small amounts of the dye return to the blood via the lymph in one hour, the per cent disappearance rate of dye from the blood stream is a fair index of the blood-lymph circulation rate, provided the hematocrit values remain constant throughout one hour and provided there is no drastic alteration in blood volume involving shifts of whole blood.



Conditions of generalized decreased capillary permeability should be detected accurately by this method, but conditions of increased permeability may or may not be detected. In the latter regard positive results are to be trusted, but negative results are not necessarily indicative of normal conditions.

#### DISCUSSION OF RESULTS

The fact that significant changes in blood volume and disappearance rate of dye were found together with loss in weight brings up the question of the possible direct role of anorexia and weight loss in the causation of these other phenomena.

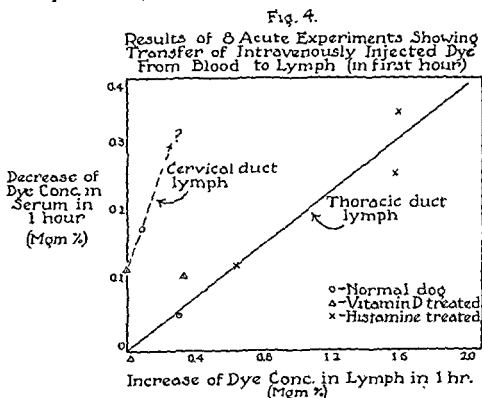
Prolonged loss of weight is accompanied by decreased total blood volume and hematocrit, according to Rowntree and Brown.<sup>7</sup> Sudden weight reduction (in obese subjects) is unaccompanied by any consistent changes in total blood



volume.<sup>8</sup> Furthermore, acidosis and dehydration specifically cause decreased total blood volume.<sup>9-11</sup> In 1937 Tislowitz and Kurowski<sup>12</sup> reported that large doses of vitamin D produced an increase in the plasma volume of circulating blood together with a decreased red cell volume. Data on one experiment only were given and no mention was made of weight changes. Doses of vitamin D administered were about  $\frac{1}{10}$  of the amount used in this work.

There are two main possibilities to account for the increased blood volume and decreased rate of dye loss found both in normal dogs and in histamine-treated dogs after vitamin D administration. They are (a) decreased capillary permeability and (b) action on the liver. Decreased capillary permeability to water and water-soluble substances could theoretically be effected by:

1. An increased amount of lipoid in endothelial cell membranes brought about by a high blood concentration of lipoid (plus dispersing agent). (The preparation of vitamin D used in this work contains a good deal of sterol, and the blood itself normally contains substances which can disperse lipoids, so that this mechanism for effecting a decrease in capillary permeability was present in our experiments.)



2. Increased concentration of calcium ions can cause decreased permeability of cellular membranes,<sup>13-16</sup> and a high blood calcium, at least, frequently follows high dosing with vitamin D.<sup>17</sup> Decreased capillary permeability would result in an increased retention of plasma proteins and therefore of fluid in the circulating blood. The dye particles, attached to plasma proteins, would also escape less easily, giving rise to a decreased dye disappearance rate.

There is also the possibility that the effects are due to changes in the circulation through the liver. As was noted by Mautner and Pick,<sup>18</sup> and later by Bauer et al.,<sup>19</sup> histamine and other so-called "shock poisons" cause constriction of the hepatic veins near the entrance into the vena cava, resulting in swelling of the liver and great outpouring of liver lymph. Other agents have been found to act oppositely; i.e., cause partial constriction of both hepatic artery and portal vein, resulting in an increased total circulating blood

volume. Vitamin D in large amounts may be one of the latter agents which "contract" the liver. This action would explain the decreased dye disappearance rates, the increased total blood volume, and the antagonism of the histamine effect.

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TABLE I  
BLOOD GLUCOSE LEVELS AFTER INSULIN ADMINISTRATION

	INSULIN ADMIN- ISTERED	BLOOD GLUCOSE MG. PER 100 C.C. AT TIMES								CONDITION ON TAKING LAST BLOOD SAMPLE	
		BEFORE	20 MIN.	30 MIN.	60 MIN.	90 MIN.	120 MIN.	180 MIN.			
Normal patient*	400 U. intramuscular	105		100	38	14				Stupor	
Average of 7 normals*	75-500 U. intramuscular	102							18	Stupor	
Resistant patient	400 U. intramuscular	129		120	60	36	30	26		Awake	
Resistant patient	940 U. intramuscular	130			71	36	27	26		Awake at 120 min. Very light stupor at 150 min.	
Resistant patient	960 U. intramuscular	130						29		Light stupor	
Resistant patient	1200 U. intramuscular	104		99	47	27	17	17		Awake	
Resistant patient	20 U. intravenous	123		89	47		31	30		Awake	
Resistant patient	400 U. intravenous	120	110		50		27	27		Awake	
Resistant patient	1500 U. intravenous	125	130		64		28	28		Awake	
Resistant patient	2500 U. intravenous	106			45			24		Awake, twitching	
Resistant patient	120 U. protamine-zinc in- sulin 12 hr. previously	104			34			25		Light stupor; awakened by a convulsion	
	500 U. insulin intravenous										

\*By "normal" is meant a patient who shows the usual stupor-producing effect of high insulin dosage.

travenous dose from 20 U. to 2,500 U., had no appreciable effect on the rate of fall in blood sugar.

The fasting blood sugar of the patient before insulin injection was nearly always higher than normal though there was no sign of glucose in the urine. The lowest level of blood sugar after insulin was also nearly always slightly higher than has been found with other patients undergoing insulin treatment. This suggests that the regulation of the blood sugar was somewhat unusual in the resistant patient. While insulin administration produced a normal total drop in blood sugar, a certain amount of sugar was not subject to regulation by insulin, and this reserve was sufficient in his case to prevent stupor. However, one low figure for the final level argues against this hypothesis. Several determinations carried out after yeast treatment indicated that no nonfermentable reducing substances were affecting the results.

#### SUMMARY

Observations are reported on a patient who received up to 1,200 units of insulin intramuscularly and up to 2,500 units intravenously without going into deep stupor. Insulin produced the same total fall in blood sugar in this patient as in patients who went into stupor.

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# PURIFIED PYROGEN FROM *EBERTHELLA TYPHOSA*\*

## A PRELIMINARY REPORT ON ITS PREPARATION AND ITS CHEMICAL AND BIOLOGIC CHARACTERISTICS

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WITH THE TECHNICAL ASSISTANCE OF ARTHUR WALLEN AND LILLY SCHMIDT

IN A PREVIOUS report from this laboratory it was stated that the fever-producing principle, or pyrogen, in typhoid vaccine is not removed by Berkefeld filtration; i.e., it is soluble and not bound to the bacterial bodies; it is retained by a 200-second Zsigmondy ultrafilter and is, therefore, of the same order of particle size as the fever-producing principle found in intravenous fluids; and like the pyrogen found in insulin and infusion fluids, it is removable by filtration through asbestos pads of the Ertel pyrogen retentive type. For these reasons, and for the reason that the clinical response provoked by an intravenous injection of "pyrogenic" fluids is similar to that provoked by typhoid vaccine, it was concluded that the fever-producing principle associated with *Bacillus typhosus* and pyrogen are closely related substances.

The present work deals with isolation of this pyrogen from typhoid bacilli and with some of its chemical and biologic characteristics.

*Method.*—The starting material was a concentrated stock suspension of killed *Eberthella typhosa* containing 50 to 88 million microorganisms per c.c. The entire procedure from beginning to the final product was carried out under aseptic precautions, all the apparatus having been autoclaved or boiled in pyrogen-free water and all the distilled water boiled fresh.

The suspension (usually from 6 to 7 liters) was centrifuged at over 3000 revolutions per minute until the supernatant was clear. The pooled supernatant fluid was passed through a Berkefeld or Mandler filter of the smallest porosity. The clear sparkling filtrate was then poured into 10 volumes of filtered 95 per cent or absolute alcohol, to which was added sodium acetate crystals in the approximate concentration of 0.5 per cent. The precipitate, which first appeared as a thick cloud, was allowed to settle over 48 to 72 hours, after which the alcoholic supernatant was siphoned off. The alcohol-precipitate mixture at the bottom of the vessel was then centrifuged and the supernatant alcohol drained off.

The grayish white precipitate, which now contained the pyrogen, was suspended with hot distilled water until a viscous suspension was obtained. It was then transferred to a Visking cellophane sac; toluene was added as a preservative. Both ends of the sac were tightly tied off, and the suspension dialyzed for 4 to 5 days in a refrigerator, first against tap, then against distilled water.

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Most of the salts were removed in this way. After dialysis, any insoluble material within the sac was centrifuged off; and to the cold supernatant were added 10 volumes of cold absolute alcohol. The resulting precipitate was centrifuged, dissolved in a minimum amount of hot water, and added to 6 volumes of 95 per cent phenol, according to the method of Palmer and Gerlough.<sup>2</sup> The concentrated phenol extracted most of the protein present. This step was repeated. The final precipitate was washed with alcohol several times to remove phenol, dissolved in a minimum amount of hot water, and poured into 10 volumes of absolute acetone. The white precipitate was centrifuged off, dried, and weighed.

In following the procedure described above, the pyrogen-containing precipitate was dried and weighed at each stage in the process of concentration and purification, and micro-Kjeldahl nitrogen determinations, according to the method of Levy and Palmer,<sup>3</sup> were carried out on samples. In this way, the loss of nitrogen could be traced.

*Physical and Chemical Characteristics.*—The final yield from 6 liters is slightly more than three Gm., or approximately half a gram per liter of the suspension. The product is a gummy substance. Its true solubility has not been determined, since it forms colloidal solutions exhibiting opalescence. The chemical properties are summarized in Table I.

TABLE I  
CHEMICAL PROPERTIES OF TYPHOID PYROGEN

TESTS	RESULTS
Molisch	Strongly positive
Pentose	Negative
Galactose	Negative
Uronic acid	Negative
Mucic acid	Negative
Reducing sugars	Negative (before hydrolysis); 30% $\pm$ after
Osazones	Melting point: 179 to 183° C., lowered by mixing with glactasazone
Millon	Negative
Biuret	Negative
Pyrogenic activity	Lost after hydrolysis

Table II embodies the elemental analyses of pyrogen prepared from the two different suspensions of *Eberthella typhosa*. It will be noted that the percentage of nitrogen is identical in both samples, being 1.5 per cent each, that the sample has a higher percentage of carbon, and that the percentage of hydrogen and ash is approximately the same. The atomic ratios are as follows:

C	39.4	30
	39.3	
	46.7	
	7.1	
H	7.0	66
	6.7	
	6.3	
N	1.5	1

The C:H:N ratio suggests that the product pyrogen is not a protein. It would be consistent with 1 glucosamine per 5 or 6 hexose units. The C:H ratio is roughly that of a polysaccharide.

The P:S:N, which is roughly 1:2:5, suggests strongly that S and P are present as impurities, since only one S and only two P occur for every 150 atoms of C.

**Minimum Pyrogenic Dose:** The MPD is arbitrarily defined as that dose which upon intravenous injection per kilogram test animal will provoke a rise of temperature of 0.5° to 0.6° C. within four after injection. Table III summarizes the MPD doses in three species of test animals, namely rabbit, dog, and man.

In a previous report,<sup>4</sup> pyrogen "A," a cruder product containing 2.5 per cent N, was used for the calibration of Ertel pyrogen retentive pads. The MPD of this product was 0.1 microgram for rabbits, 0.4 microgram for dogs, and 0.3 microgram for men. This makes the rabbits approximately three times, and the dog 12 times less sensitive than man. In the present, more highly purified preparation, this approximate relationship still holds (See Table III).

TABLE II  
ELEMENTAL ANALYSIS OF TYPHOID PYROGEN

STRAIN*	N	C	H	S	P	ASH
Squibb	1.5	39.4	7.1	1.25	0.61	4.4
		39.3	7.0	1.29	0.67	4.5
Lilly*	1.5	46.7	6.7			4.2

\*The Lilly material contained equal parts of (1) Army Medical School, Panama, No. 58 strain, and (2) Army Medical School, Rawling, No. "N" strain. The Squibb material contained Panama No. 58 strain.

TABLE III  
MINIMUM PYROGENIC DOSES OF TYPHOID PYROGEN

EXPERIMENTAL ANIMALS	NO. USED	DOSE IN MICROGRAMS PER KILOGRAM ANIMAL
Rabbit	8	0.06
Dog	8	0.24
Man	6	0.02

The approximate minimum lethal dose of this purified typhoid pyrogen on the rabbit has been determined and is summarized in Table IV. It lies between 175 and 190 micrograms per kilogram rabbit.

It will be seen that the spread between the MPD and the minimum lethal dose is rather high, over three thousand times. The rabbits receiving the lethal doses die within two hours, in convulsions, with only moderately elevated temperatures. It is quite possible that had the rabbits lived longer, a higher body temperature might have developed.

Favorite and Morgan<sup>5</sup> reported in 1942 that 100 micrograms of their preparation of antigenic material from *Eberthella typhosa* in man caused a temperature rise of from 100.2° to 101.8° F. This effect is almost within the range of our definition for the effect provoked by our MPD. Since our present material gives this rise in doses of 0.02 microgram per kilogram, or 1.2 micrograms for a 60-kilogram man, it appears that the present material is almost a hundred times more potent than that of Favorite and Morgan, which, according to one of their reports<sup>6</sup> contains 7 per cent of nitrogen.



It will also be pertinent to record the case of a patient who received 15 micrograms of this purified preparation per kilogram, i.e., 750 times the MPD.

She weighed 78.6 kilograms, with initial leucocyte count of 10,300, and rectal temperature of 37.5° C., a pulse rate of 96, a respiration rate of 20, and blood pressure of 123/80. At 2:45 P.M., she was given the injection. In one hour and fifteen minutes the temperature had reached 40° C., the pulse rate was first slightly lowered, then rose to the initial level, and the respiration was only slightly accelerated. The leucocyte count at this time was 6,200, a suggestive leucopenia. At 8:25 P.M., her temperature had risen to 40.9° C., pulse rate to 140, and respiration to 28. She vomited and was incontinent; her blood pressure was 70/50. She became unconscious and stayed unconscious for 48 hours. In 24 hours, her blood pressure was 110/70, her temperature was still 40.5°, and her pulse rate was 128. Her temperature reached normal in 72 hours. If this dose is near fatal, then the spread between the minimum lethal and the minimum effective dose in man would be in the order of hundreds and not thousands as it is in rabbits.

No immunologic studies have been done with pyrogen, for the reason that we are not yet assured of its purity.

TABLE IV  
MINIMUM LETHAL DOSE OF TYPHOID PYROGEN

DOSE IN MICROGRAMS PER KILOGRAM RABBIT	NO. OF ANIMALS USED	NO. SURVIVING
150	3	3/0
179	3	2/1
190	3	0/3

TABLE V  
COMPARISON OF TYPHOID PYROGEN AND RELATED PRODUCTS

AUTHORS	YEAR	REF. NO.	RAW MATERIAL	NAME OF SUBSTANCE	METHOD OF PREPARATION	ELEMENTARY ANALYSIS		
						N PER CENT	REDUC- ING SUGARS PER CENT	P PER CENT
Topley, et al.	1937	7	Bacterial bod- ies	Antigenic material	Tryptic digestion	4.8- 5.3		
Henderson and Morgan	1938	8				2.25- 2.4		1.5-2
Dennis, et al.	1939	10	Cells	Typhoid Leuco- cidin	Trichlo. acetic acid	4.5		4.6
Freeman, et al.	1940	9	Cells		Tryptic digestion Trichloroacetic acid Ethylene glycol	4.2- 7.2	32-46	
Morgan	1941	6	Growth, syn- thetic me- dium	Antigenic material	Sodium acetate and alcohol precipitation	7		5.6
Co Tui et al.	1943	Pres- ent work	Supernatant of concen- trated ty- phoid sus- pension	Pyrogen	Sodium acetate, al- cohol precipita- tion, acetone reprecipitation	1.5	30%	0.61- 0.67

## COMMENTS

A review of the works of Topley et al.,<sup>7</sup> Freeman et al.,<sup>9</sup> Henderson and Morgan,<sup>8</sup> Morgan,<sup>6</sup> and Dennis and Senekjian<sup>10</sup> on typhoid antigens and typhoid leucocidin brings out striking similarities of these substances with the present product.

Chemically, it behaves like the antigens in question: It contains a polysaccharide, and it splits into reducing sugars after hydrolysis and forms osazones. Its elemental composition, particularly in N content, does not differ too greatly from that of these antigens, in view of the crudity of these various preparations, the differences being attributable to the different methods of preparation (see Table V for a compilation of the methods and the figures of elemental analyses). Finally, both pyrogen and these antigens are highly potent chemically, evoking temperature and leucopenia in microgram doses.

## SUMMARY

1. A fever-producing principle or pyrogen from the *Eberthella typhosa* has been isolated and purified.
2. Some of its chemical characteristics are studied.
3. A tentative attempt is made to identify it with the typhoid antigens isolated from the *Eberthella typhosa* by other workers.

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# LABORATORY METHODS

## GENERAL

### THE REAPPEARANCE OF FLICKER AT HIGH FLASH FREQUENCY IN PATIENTS WITH BRAIN PATHOLOGY AND IN NORMAL SUBJECTS\*

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#### INTRODUCTION

**D**URING the course of experiments on the fusion frequency of flicker,<sup>1-5</sup> a second and a third reappearance of flicker was observed when the speed of the motor was increased beyond the fusion frequency of the original or first flicker. This reappearance is conveniently named the "second" and "third" flickers respectively. Beginning with a frequency of about 105 flashes per second, a faint flicker appears again. With further increase of flash frequency the flicker becomes more and more manifest and reaches a maximum of intensity at about 115 flashes per second. Finally, the flicker disappears at a frequency of about 122. After a short interval of two to five flashes per second, a third flicker appears; this reaches its maximum intensity between 130 and 140 and then disappears between 145 and 150. After this, no further flicker was detected.

The second and third reappearance of flicker occurs only when alternating current is used and not with direct current. Therefore, it must be regarded as an interference phenomenon between the waves of luminosity of the filament, produced by the cycles of the alternating current and the light flashes. It is an objective phenomenon, for it can be photographed directly by means of a movie camera or indirectly by means of a photoelectric cell and a cathode ray oscillograph. The second and third flickers are less distinct with a thicker lamp filament, because the waves of luminosity are more level. The second and third flickers are most manifest with a Neon bulb, because with this light source there is practically no inertia. Early in these investigations it was found that the recognition of the second and third flickers was impaired in patients with brain pathology and usually this was more marked than the impairment of their recognition of the first flicker. Phillips<sup>6</sup> reported a decrease of the fusion frequency of flicker in 8 chiasmal and in 2 out of 4 parietal

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brain tumors which are independent of visual acuity and visual field. Werner<sup>9</sup> found in a group of 20 children with brain injury a pronounced depression of the fusion frequency. It seemed possible that the phenomenon of the reappearance of flicker might be worked out as a clinical method for the study of brain conditions. For this purpose, normal data for the range of the second and third flickers in three standard arrangements were established in 25 normal healthy subjects with normal vision and normal ocular structure. This material was used for comparison with a series of observations on 41 patients with various disorders of the central nervous system.

#### METHOD

We used the same rotator arrangement as in our former experiments.<sup>1-5</sup> The beam of light from a 25 watt Mazda bulb was interrupted by a rotating disk with six openings; 60 cycle alternating current was used and the voltage kept constant by a regulator. Two disks were used. In one the relationship between duration of flashes and duration of dark intervals was 50:50 (50 per cent); in the other disk it was 17.5:82.5 (17.5 per cent). Two different square exposure areas were used, with a visual angle of  $\frac{1}{2}$  degree and 1 degree respectively. Consequently, all results concern the fovea only. A few minutes' adaptation time was found to be sufficient to obtain reliable and constant results. The surrounding illumination was 2.0 foot-candles at the plane of the illuminated area and 0.32 foot-candle at the subject's eye. The time of exposure was limited to 1.5 seconds. Three different intensities of illumination were used by placing a diaphragm (1, 2, and 3) with a different size opening in front of the source of illumination.

Out of eighteen different combinations of size of area, intensity of illumination, and relative duration of flashes, investigated in two normal subjects, three standard arrangements were chosen for the investigation of 25 normal subjects and 41 patients: (1) Relative flash duration 50 per cent; diaphragm 2 (= 0.003 candle power per sq. cm.); visual angle 1 degree. (2) Relative flash duration 17.5 per cent; diaphragm 2 (= 0.001 candle power per sq. cm.); visual angle  $\frac{1}{2}$  degree. (3) Neon bulb; relative flash duration 50 per cent; no diaphragm; visual angle  $\frac{1}{2}$  degree. Each eye was examined separately. The first flicker was investigated with arrangement (1). When using arrangement (2) or (3), the first flicker disappears at a much higher flash frequency, so that the flicker-free interval is shorter.

The maximum of the second and third flickers occurred at the same flash frequency in all arrangements, but the range of frequencies, over which the second and third flickers could be seen, i.e., the difference between appearance and disappearance, varied. In this report the observations are concerned with the *range* of the second and third flickers. Therefore, measurement of the second and third flickers includes two fusion points (appearance and disappearance). The experimental error of the determination of the first flicker is about one flash per second; the error in the determination of the range of the second and third flickers is about two flashes per second. As with the first flicker, it was found impossible to affect the accuracy or range of observation of the second and third flickers by effort or training.

## RESULTS

Table I shows the limits, distribution, average and standard error of the range of the second and third flickers, established in 50 individual eye values of 25 normal subjects. The second or third flicker is easiest to recognize with the Neon arrangement (highest average value, small standard error), and hardest at 50 per cent flash duration (smallest average, highest standard error). The standard error at 17.5 per cent flash duration is about the same as that with the Neon arrangement. The higher standard error at 50 per cent flash duration means that deviations are less significant with this arrangement. On the other hand, deviations occur earliest at 50 per cent flash duration when the values might still be normal at 17.5 per cent flash duration. The lowest normal limits seem to be the most important for clinical purposes. A pathologic decrease may be assumed to be present when the value is smaller by one flash than the lowest limit at 17.5 per cent flash duration and the Neon arrangement, and by two flashes at 50 per cent flash duration.

The average range of the second flicker is somewhat greater than the average range of the third flicker, but this difference is not statistically significant. Differences between the right and left eyes were analyzed according to the data on averages, standard deviations, and distribution. A difference between the right and left eyes in the perception of the first flicker, the second and third flickers with the Neon arrangement, and at 17.5 per cent relative flash duration, can be regarded as a significant pathologic deviation, when exceeding two flashes. At 50 per cent flash duration, the normal limit of differences between the right and left eyes for the second and third flickers is three flashes. There was no evidence that sex or age influenced the perception of the second and third flickers.

Tables II to VI give a schematic summary of the fusion of flicker findings in 41 cases, grouped according to their clinical diagnosis. The vision was normal if not stated otherwise. Column 2 of Tables II to VI shows the date when the fusion frequency was tested.

## GROUP I, BRAIN TUMOR

1. C. F., male, aged 29. Inoperable left frontal lobe tumor.
2. B. W., female, aged 40. Glioblastoma of left frontal temporal area. Resected 1/24/40; followed by x-ray therapy. 3/3/41 recurrent tumor in same area removed, followed by x-ray therapy.
3. O. O., male, aged 47. Glioblastoma over corpus callosum removed. X-ray therapy.
4. R. S., female, aged 36. Diagnosis of petit mal (September, 1940). April, 1941, operation for left parietal astrocytoma. X-ray therapy.
5. A. R., male, aged 34. Left frontal tumor removed. X-ray therapy.
6. P. H., male, aged 46. Tumor of floor of third ventricle (infundibuloma).

The results of patients of Group I are shown in Table II. For convenience, the lowest normal limits, taken from Table I, are added in the last line.

All patients show complete abolition (0) or pronounced reduction of the range of the second and third flickers in one or both eyes at 50 per cent flash duration and abolition, pronounced or moderate reduction at 17.5 per cent flash duration. In Patient 5 the range of the second flicker at 17.5 per cent flash duration coincides with the lowest normal limit, while the third flicker is moderately reduced on the right and slightly reduced on the left eye. There are



significant differences between the right and left eyes in Patients 1 to 5 for the second and third flicker, and in Patients 1, 3, 4 also for the first flicker. The first flicker is depressed in Patients 4 and 6 (both eyes) and in Patient 3 on the left eye only. In Patient 4 the recognition of flicker was impaired four months before the first definite neurological findings were obtained to warrant a competent neurologist to recommend surgery. Also in Patient 6 the fusion frequency showed pronounced depression (first flicker) and complete abolition of the second and third flickers at a time when the neurological findings failed to give clear evidence. Interesting is the improvement of the recognition of the second and third flickers in Patient 1 after x-ray therapy, paralleling subjective improvement. In Patients 2, 3, and 5 the flicker test was performed after recovery from operative removal of the tumor. In these cases the reduction of the flicker is related to the effects of both tumor and operation. Since these cases show a pronounced deviation in the presence of considerable clinical improvement, it seems safe to assume that the deviations were not less before surgery.

## GROUP II. PARKINSON'S DISEASE

7. C. S., female, aged 14. Postencephalitic parkinsonism.
8. E. R., male, aged 36. Postencephalitic parkinsonism.
9. E. L., male, aged 43. Postencephalitic parkinsonism.
10. M. N., male, aged 42. Postencephalitic parkinsonism.
11. C. S., female, aged 62. Mild parkinsonism. No history of encephalitis.

TABLE III

THE RANGE OF THE SECOND AND THIRD FLICKERS IN PATIENTS WITH PARKINSON'S DISEASE

CASE NO.	DATE	FIRST FLICKER	50% FLASH DURATION FLICKER				17.5% FLASH DURATION FLICKER				NEON ARRANGEMENT FLICKER			
			SECOND		THIRD		SECOND		THIRD		SECOND		THIRD	
			R	L	R	L	R	L	R	L	R	L	R	L
7	9/14/40	42.0	42.0	0	0	0	0	0	0	0				
	2/18/41	41.6	41.6	0	0	0	0	0	0	0	9.0	9.0	7.2	7.2
8	10/24/40	39.2	42.6	0	0	0	0	3.8	11.2	7.2	10.8			
	6/22/41	41.4	43.2	3.2	7.6	4.4	6.8	11.4	13.8	6.0	8.4*			
9	5/25/41	40.2	43.2	4.2	4.2	3.0	6.0	7.2	11.4	11.4	11.4	11.4	10.2	9.6
10	6/14/41	48.4	49.2	6.8	10.8	6.0	7.8	9.6	13.8	7.2	9.6			
11	9/10/41	42.0	42.0	0	0	0	0	12.6	7.2	10.2	7.2	12.6	11.4	10.8
	9/10/41	42.0	42.0	0	0	0	0	13.8	13.8	10.8	10.8	15.0	15.0	13.2
	Lowest normal limits	42.0	42.0	10.8	10.8	9.0	9.0							

\*Some subjective improvement.

The results of this group are shown in Table III. The first flicker is near the lowest normal limit except in Patient 10, who has values within the normal range. At 50 per cent flash duration, recognition of the second and third flickers was abolished (Patients 7, 8, and 11) or markedly depressed. There was a moderate reduction, at least in one eye, at 17.5 per cent flash duration, and with the Neon arrangement in Patients 8 to 11, while Patient 7 showed pronounced reduction with the Neon arrangement and complete abolition with 17.5 per cent flash duration. Significant differences in the recognition of the second and third flickers between the right and left eyes were observed in Patients 8, 7, and 10, although they are not consistent in all arrangements in Case 9. The second examination of Patient 8, performed eight months after the first examination, showed an increase of the range of the second flicker, paralleling a subjective improvement.

## GROUP III, HEAD INJURIES

12. J. C., male, aged 62. Unconscious after injury. Headache, vertigo, and weakness.
  13. E. H., male, aged 54. Unconscious after injury. Headache and vertigo.
  14. J. E., male, aged 36. Skull fracture. Unconscious after injury. Complete loss of hearing of right ear, secondary right optic nerve atrophy. Vertigo, intermittent headache, spastic gait, right hemiparesis.
  15. F. B., male, aged 31. Not unconscious after injury. Headache, vertigo, vestibular imbalance, and slight nystagmus.
  16. W. D., male aged 21. Unconscious after injury. Headache, vertigo, and personality change.
  17. A. S., male, aged 37. Unconscious after injury. Headache, vertigo, vestibular imbalance, loss of hearing, personality change. Normal encephalogram. Bloody fluid removed from right posterior fossa after flicker test.
  18. J. T., male, aged 22. Unconscious after injury. Temporal lobe hemorrhage. Recovery after surgery. Personality change.
  19. R. F., male, aged 18. Not unconscious after injury. Jacksonian epilepsy. Improvement after surgery, March, 1940.
  20. K. M., male, aged 51. Unconscious after injury. Headache. Vision 15/30.
  21. R. K., female, aged 29. Not unconscious after injury. Vertigo, headache, disturbed gait, and vestibular imbalance.
  22. C. Z., male, aged 50. Skull fracture. Unconscious after injury. Headache, vertigo, and vestibular imbalance.
  23. J. B., male, aged 54. Unconscious after injury. Vestibular imbalance, loss of hearing, headache, and mental deterioration.
  24. R. C., female, aged 19. Unconscious after injury. Headache, vertigo, and vestibular imbalance.
  25. R. G., male, aged 33. Not unconscious after injury. Headache, vertigo, and vestibular imbalance.
  26. C. E., male, aged 21. Unconscious after injury. Headache, vertigo, and vestibular imbalance.
  27. H. J., female, aged 19. Skull fracture. Unconscious after injury. Headache, vertigo, and vestibular imbalance. Hearing loss, left ear.
  28. H. W., male, aged 40. Not unconscious after injury. Vertigo. Quick recovery.
- (The dates of injury and flicker test for above patients are shown in Table IV.)

Table IV shows the results of these seventeen patients with postconcussion syndrome. The clinical objective data in most of these cases are meager, in contrast to the pronounced complaints. In Case 22 the second and third flickers were abolished or greatly reduced eight weeks after the skull fracture, when all neurological findings were normal with the exception of increased vestibular excitability. Case 27 is similar. The results of the flicker test naturally depend on the severity of the injury, the progress of convalescence, and the interval between flicker test and injury. All cases except 17, 21, and 28 show abolition or pronounced reduction of the range of the second and third flickers at 50 per cent flash duration; abolition or pronounced to moderate reduction at 17.5 per cent flash duration; and moderate to pronounced reduction with the Neon arrangement. Patient 17, investigated six months after the accident, showed slightly decreased values at 17.5 per cent flash duration and with the Neon arrangement. Patient 21 had resumed her work several weeks before the flicker test. Patient 28 had suffered the slightest injury of all cases of this group; his values were normal, but near the lowest limit at the first examination; there was a definite increase in all arrangements three weeks later, when the patient felt completely recovered again. Since the normal values of



the patient are not known, low normal values do not exclude a pathologic reduction. An improvement of the recognition of the second and third flickers at the second examination is shown in Patient 26 after an interval of two months (with the Neon arrangement), in Patient 12 (interval of one month), and Patient 16 with an interval of three days only. The majority of patients show significant differences of the second and third flickers between the right and left eye. Nine of 17 patients show also depression of the first flicker, and out of eight with normal values of the first flicker four have significant differences between the right and left eyes.

## GROUP IV, MISCELLANEOUS CASES

29. R. S., female, aged 42. Left hemiplegia 2/2/39. Recovered. Hypertension.  
 30. L. K., female, aged 37. Multiple sclerosis.  
 31. T. S., female, aged 31. Multiple sclerosis.  
 32. H. L., male, aged 29. Cerebral abscess drained in 1936. Ataxia and vertigo. Vision: left eye, normal, right eye with correction, 15/40.  
 33. J. H., female, aged 13. Onset of convulsions at 9 years of age. Probable Jacksonian epilepsy.

TABLE IV

THE RANGE OF THE SECOND AND THIRD FLICKERS IN PATIENTS WITH HEAD INJURIES

NO. CASE	DATE OF INJURY		FIRST EXAMI- NATION	FIRST FLICKER R L	50% FLASH DURATION FLICKER				17.5% FLASH DURATION FLICKER				NEON ARRANGEMENT FLICKER			
					SECOND		THIRD		SECOND		THIRD		SECOND		THIRD	
	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L
12	7/10/40	9/11/40	43.2	45.6	0	0	1.8	4.2								
		10/13/40	44.0	46.0	0	1.4	2.4	6.2								
13	May '40	7/12/41	36.0	36.0	0	0	0	0	0	0	0	0				
14	8/26/40	8/15/41	39.0	44.1	0	0	0	0	0	0	0	0	10.2	12.6	7.8	9.0
15	10/26/40	7/22/41	39.0	37.2	0	0	0	0	0	0	0	0	7.2	4.8	5.4	4.8
16	7/ 6/41	8/12/41	45.0	49.2	0	0	0	0	0	10.2	1.2	9.6	7.6	10.8	6.6	9.6
	*	8/15/41	48.6	50.4	0	1.8	0	4.8	6.0	10.2	7.8	9.6				
17	3/ 1/41	6/ 1/41	47.4	48.0	10.2	7.2	9.0	8.4	10.8	7.2	9.0	8.4	14.4	13.2	12.0	10.2
18	3/14/41	6/23/41	†	38.0	0	10.8	0	9.6	0	11.4	0	10.8	0	16.8	0	15.0
19	Child- hood	7/14/41	39.0	39.0	4.2	0	1.8	0	6.6	6.6	8.4	6.6	13.2	13.2	12.0	10.2
20	7/15/39	9/11/41	35.4	33.0	0	0	0	0	7.2	7.2	7.2	7.2	9.0	6.0	7.2	2.4
21	5/31/41	10/11/41	50.4	51.6	10.8	15.0	10.2	10.2	15.6	13.8	14.4	15.6				
22	8/21/41	10/14/41	43.2	44.4	0	0	0	0	3.0	1.8	2.4	0	4.8	2.4	3.6	1.8
23	8/ 4/39	10/14/41	38.4	42.0	0	8.4	0	8.4	0	12.0	4.2	7.8	6.6	11.4	6.0	8.4
24	1/10/42	2/17/42	36.6	36.6	0	0	0	0	6.6	4.8	6.6	5.4				
25	9/12/41	1/10/42	34.8	37.2	0	0	0	0	0	0	0	0	1.8	6.0	3.0	6.0
26	8/11/41	10/23/41	40.8	45.0	0	0	0	0	0	0	0	0	7.8	13.8	8.4	9.0
		12/19/41	42.6	45.6	0	0	0	0	0	0	0	0	13.8	14.4	10.2	9.6
27	8/18/41	1/10/42	43.8	46.8	0	1.8	5.4	4.2	11.4	12.6	13.2	15.0				
28	1/ 9/42	1/17/42	42.6	42.6	12.0	13.8	12.0	12.6	12.6	9.6	12.0	12.0				
		2/ 7/42	45.6	46.8	15.0	15.0	16.2	15.6	14.4	13.2	15.0	13.8				
Lowest normal limits			42.0	42.0	10.8	10.8	9.0	9.0	13.8	13.8	10.8	10.8	15.0	15.0	13.2	13.2

\*Spinal puncture on August 14

†Patient had difficulty to discriminate the fusion of first flicker with right eye.

Table V shows the results of Group IV. The recognition of the second and third flickers is impaired in all patients, while the first flicker is depressed in two patients (29 and 31).

## GROUP V, NEUROLOGICAL SYMPTOMS WITHOUT DEFINITE DIAGNOSIS

34. J. C., male, aged 36. Diabetic. Nerve deafness. Right eyelid ptosis. Right external ophthalmoplegia. Paralysis of inferior rectus and internal rectus muscles. Spinal fluid protein increased. Diplopia.

35. D. U., male, aged 55. Loss of superficial and deep sensation. Left arm. Astereognosis. Sclerosis of retinal vessels. Probably vascular disease right brain.
36. S., male, aged 31. Spastic gait. Impaired coordination. Loss of weight. Personality change. Diagnosis: possible brain tumor.
37. E. R., male, aged 26. Recurrent vertigo. Paresthesia. Transient hemiparesis. Left temporal pain. Normal encephalogram.
38. S. W., male, aged 30. Weakness and numbness of left hand. Numbness of left thigh. Hemiparesis. Left tendon reflex absent.
39. R. B., male, aged 32. Headache. Epileptiform seizures. Onset three months after injury. Vision: right eye, normal, left eye, 15/100.
40. P. R., male, aged 54. Head injury 2/2/40. Not unconscious. Vertigo. Injured area painful. Increased vertigo.
41. A. B., female, aged 38. Recurrent attacks of syncope. Widely spread, generalized convulsions. Probable epilepsy. Encephalogram was normal.

TABLE V

THE RANGE OF THE SECOND AND THIRD FLICKERS IN PATIENTS WITH MISCELLANEOUS DISORDERS

CASE NO.	DIAGNOSIS	DATE	FIRST FLICKER		50% FLASH DURATION FLICKER				17.5% FLASH DURATION FLICKER				NEON ARRANGEMENT FLICKER			
					SECOND		THIRD		SECOND		THIRD		SECOND		THIRD	
			R	L	R	L	R	L	R	L	R	L	R	L	R	L
29	Hemiplegia	9/24/40	32	33	0	0	0	1.8								
30	Multiple sclerosis	5/17/41	48.4	*	1.8		0		4.2		4.2		7.8		8.4	
31	Multiple sclerosis	12/18/42	30.6	25.8	0	0	0	0	0	0	0	0	6.0	6.6	6.0	6.6
32	Cerebellar abscess	10/ 7/41	45.8	46.4	0	8.4	0	7.2	0	10.2	7.8	6.6	9.6	12.0	10.2	11.4
33	Jacksonian epilepsy	8/15/41	45.6	45.6	0	0	0	0	0	0	0	0	11.4	10.8	9.6	12.6
Lowest normal limits			42.0	42.0	10.8	10.8	9.0	9.0	13.8	13.8	10.8	10.8	15.0	15.0	13.2	13.2

\*It was impossible to determine the flicker for the left eye because of interference of visual sensations.

TABLE VI

THE RANGE OF THE SECOND AND THIRD FLICKERS IN PATIENTS WITH NEUROLOGICAL COMPLAINTS BUT WITHOUT DEFINITE DIAGNOSIS

CASE NO.	DATE	FIRST FLICKER		50% FLASH DURATION FLICKER				17.5% FLASH DURATION FLICKER				NEON ARRANGEMENT FLICKER			
				SECOND		THIRD		SECOND		THIRD		SECOND		THIRD	
		R	L	R	L	R	L	R	L	R	L	R	L	R	L
34	8/14/41	36.6	38.4	4.2	6.0	3.6	5.4	7.8	9.0	5.4	9.0	10.2	11.4	9.6	10.8
35	8/17/41	45.6	43.8	7.2	0	6.6	0	12.0	7.8	10.2	7.8	13.2	8.4	11.4	7.2
36	7/25/41	43.8	44.4	0	0	0	0	16.2	7.8	11.4	9.0	17.4	9.6	15.0	9.0
37	7/11/41	45.0	49.8	8.4	7.2	5.4	9.0	13.2	12.6	10.2	15.6	15.0	15.0	11.4	11.4
38	8/23/41	46.4	45.8	9.6	8.4	9.6	8.4	16.2	14.4	16.2	13.2	17.4	15.0	11.4	9.6
39	7/11/41	48.6	49.8	13.8	7.2	15.0	6.0	17.4	9.6	18.0	11.4	14.4	11.4	13.8	12.6
	9/13/41	49.6	51.0	15.0	12.0	18.0	10.2	18.0	14.4	18.0	14.4	14.4	14.4	13.8	13.8
40	8/11/41	40.2	40.8	9.0	13.2	6.0	10.8	10.8	10.2	11.4	15.0				
41	7/11/41	43.2	43.2	10.8	7.8	12.6	8.4	13.8	14.4	13.2	13.2	16.8	14.4	12.6	13.2
Lowest normal limit		42.0	42.0	10.8	10.8	9.0	9.0	13.8	13.8	10.8	10.8	15.0	15.0	13.2	13.2

\*Some subjective improvement.

Table VI shows the results of patients of this group. The impairment of the recognition of the second and third flickers is more pronounced in Cases 34 to 36 than in Cases 37 to 41, which fact appears to agree with the clinical history. In Cases 39, 40, and 41 an organic lesion was not considered likely.

The injury, which Patients 39 and 40 had sustained two and three years prior to the examination, was so slight that it could hardly account for the complaints, so that we did not feel that these cases should be placed in Group III (head injuries). In Case 38 all values were within the normal range, and the only deviation was a significant difference between the left and right eyes. The same was true for Patient 39 after recovery from an epileptic attack. The second examination, performed a few weeks after the attack, revealed an increase of the range of the second and third flickers and a diminution of the difference between the right and left eyes, compared to the first examination performed a few days after the attack. In this patient, the first flicker was normal and almost equal for both eyes, in spite of the different visual acuity. In general, the deviations of this group are less pronounced than those of Groups I to IV with definite brain pathology. However, Cases 4 and 6 belonged to the group of patients without definite diagnosis until more definite findings appeared. Systematic observations of cases without definite diagnosis will help to evaluate the clinical importance of this procedure.

TABLE VII  
SUMMARY OF RESULTS

GROUP	TOTAL NUMBER OF CASES	FIRST FLICKER DECREASE	SECOND AND THIRD FLICKERS		NO CHANGES OR NEAR THE LOWEST NORMAL LIMIT	SIGNIFICANT DIFFER- ENCES BETWEEN RIGHT AND LEFT EYE FLICKER	
			ABOLI- TION OR PRO- NOUNCED REDUC- TION	SLIGHT REDUC- TION		FIRST	SECOND AND THIRD
Brain Tumor	6	3	5	1	0	3	4
Parkinson's Disease	5	1	4	1	0	2	3
Head Injury	17	9	13	2	2	10	9
Miscellaneous Disorders	5	2	5	0	0	1	1
Total	33	15 (45.5%)	27 (82%)	4 (12%)	2 (6.1%)	16 (48.5%)	17 (51.5%)

#### DISCUSSION

Table VII shows the summary of results in 33 patients with definite brain pathology (Groups I to IV). Twenty-seven or 82 per cent show abolition or pronounced reduction on one or both eyes. In 31 or 94 per cent the values are lower than the lowest normal limit. The error, computed according to the formula  $\sqrt{\frac{p_1 \text{ per cent} \cdot p_2 \text{ per cent}}{n}}$  where  $p_2 \text{ per cent} = 100 - p_1 \text{ per cent}$  (Poll<sup>6</sup>), is 4.144. Since statistical reliability is regarded to be three times this value, it can be predicted that the range of the second and third flickers will be below the lowest normal limit between 81 and 100 per cent of patients with definite brain pathology. Therefore, examination of the second and third flickers may contribute additional information to the clinical data, which are often not conclusive. This seems especially true for patients with postconcussion syndrome (Group III). The fact that most cases with organic lesions exhibit a reduction of the second and third flickers does not exclude serious functional disturbances, such as epileptic attacks, as the cause of a similar reduction, as for example, Case 39. On the other hand, every lesion of the

brain need not cause impairment of the recognition of the second and third flickers. The nature and location of the lesion, and to a certain degree individual reaction of the patient, undoubtedly are important determining factors.

The second and third flickers are abolished earliest at 50 per cent relative flash duration. Twenty-seven out of 33 patients could not see the second and third flickers with one or both eyes. At 17.5 per cent flash duration, 14 patients (Cases 3, 6, 7, 13, 14, 15, 16, 18, 22, 25, 26, 31, 32, and 33) were not able to see the second and third flickers with one or both eyes. With the Neon arrangement, where the second and third flickers are most manifest, its recognition was abolished in two patients only (3 and 18). Thus, the degree of impairment in the recognition of the second and third flickers can be investigated by comparison of the three different arrangements.

In five cases (1, 8, 12, 16, and 28) clinical improvement was accompanied by an improved ability to recognize the second and third flickers.

The fusion frequency of the first flicker was decreased in only 15 out of 33 patients (45.5 per cent) on one or both eyes. In all these patients the recognition of the second and third flickers was abolished or markedly reduced. But in the other patients with definite brain pathology the second and third flickers were reduced in spite of normal values of the first flicker, with the exception of Cases 21 and 28, who had values within the normal range. In the eight patients without definite diagnosis (Group V) the fusion frequency of the first flicker was decreased only in case 34. It appears that the perception of the second and third flickers is more sensitive to brain lesions than the first flicker. In Patients 10, 16, 17, and 30 high normal values of the first flicker are associated with marked reduction of the second and third flickers. This suggests that the perception of the second and third flickers might be due to processes other than the perception of the first flicker. This is supported by the response to increase of light intensity, which always produces over a wide range of intensities (such as used in our experiments) an increase of the first fusion frequency in normals as well as in patients, while it fails to increase the range of the second and third flickers.

In 17 of 33 patients with definite brain lesions there were significant differences of the range of the second and third flickers between the right and left eyes. Comparison with the clinical data show that cerebral lesions, if localized on one side, usually decrease the function of the visual center of the opposite side, although to a lesser degree than the depression of the center on the same side. It appears that there is a general disturbance of cerebral functions associated with local cerebral lesions. This general effect disappeared with improvement of the condition of Patient 1 after x-ray therapy. As a rule the reduction of the range of the second or third flickers was more pronounced on the side opposite the cerebral lesion, except for some cases with frontal lobe lesions. This would agree with Elsberg and Spotnitz<sup>17</sup> observations, made by means of another visual method (refractory period of adaptation).

While further investigations of the nature of the second and third flickers are desirable, the clinical importance of this phenomenon can only be demonstrated by comparison of normal subjects with patients in standard conditions. The accuracy with which the phenomenon can be reproduced depends: (1) on

the reproduction of size of area illuminated, (2) surrounding illumination, (3) illumination of the test patch, (4) relative duration of flashes, and (5) constancy of the cycles of the alternating current. Reproduction of conditions (1) to (4) is easy; the data are given in this paper. A deviation of the intensity of illumination of  $\pm 20$  per cent would not influence the results. We have inquired at the Wisconsin Electric Power Company in regard to the constancy of the cycles of the alternating current. They report that the variation from 60 cycles does not exceed a few hundredths of one per cent in Milwaukee as well as in other cities of this country using 60 cycles A.C. The same phenomenon could be obtained also with any other frequency of cycles, but in this case new normal standards would be necessary. The use of the same type of electric bulb (25 watt Mazda), which can be regarded as standardized, is also important.

#### SUMMARY

When using alternating current and increasing the frequency of light flashes in a rotator arrangement beyond the fusion frequency of the first flicker, a second flicker appears at a higher frequency and, after its disappearance, a third flicker appears at a yet higher frequency. It is an objective phenomenon, for it can be photographed. It is due to interference of light flashes with fluctuations of luminosity. In 31 of 33 patients with cerebral lesions the recognition of the second and third flickers was abolished or greatly reduced as compared with the lowest limit of 25 normal subjects. In two patients the reduction of the second and third flickers was noted before any other indicative neurological findings could be obtained, and in another patient there was a marked reduction eight weeks after head injury when the neurological findings were considered normal. Clinical improvement in five patients was accompanied with improved recognition of the second and third flickers. Investigation of eight patients with nervous symptoms but without definite diagnosis of cerebral lesion revealed lesser deviations.

It is a pleasure to acknowledge our surgeon and Professor of Neurosurgery at (Attending Neurologist and Assistant Clinician, Medical School) and Dr. M. S. Fox (Attending Neurologist, Dr. S. Pollack, Marquette University) and most of the cases.

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## QUICK PARAFFIN METHOD FOR SMALL BIOPSIES\*

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OCCASIONALLY there is need for a quick microscopic examination of biopsy fragments too minute for satisfactory preparation of frozen sections. It has been found that satisfactory paraffin sections may be obtained within an hour by the following procedure:

1. Fix in 4 per cent formalin or in Zenker's fluid at room temperature for ten minutes. After Zenker fixation, wash in several changes of tap water.

2. Stain in any hematoxylin used routinely for one minute so that the pieces may be more readily seen in the block.

3. Wash in tap water.

4. Dehydrate in alcohol 80 per cent three minutes; 95 per cent, three minutes; absolute, three minutes. Clear in xylol, five minutes.

5. Transfer to melted paraffin at 56° C. five minutes and a second paraffin at 56° C. for five minutes.

6. Embed in paraffin.

7. Cut thin sections; transfer to slides and dry for five to ten minutes at 50° C. Cool slides.

8. Staining procedure as usual for paraffin sections. After Zenker fixation use Lugol solution as usual.

If special stains are required, the hematoxylin may be decolorized in 1 per cent acid alcohol.

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# THE LABORATORY DIAGNOSIS OF TUBERCULOSIS

## A COMPARATIVE STUDY OF ANIMAL INOCULATION AND MICROSCOPIC EXAMINATION

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IN THE past forty years there has been a tremendous decline in the death rate from tuberculosis in the United States. In 1904 the National Tuberculosis Association was formed and since that time much has been accomplished by organized effort to control tuberculosis. Many factors can account for the lowering of the annual incidence of tuberculosis cases: the organization of medical men, public health workers, sanitariums, welfare agencies, etc., and the near elimination of tuberculosis in dairy cattle and widespread pasteurization of milk in the United States. These things will not be discussed here, as we are more interested in the laboratory diagnosis of tuberculosis.

As tuberculosis has been brought under better control and cases have become fewer, its diagnosis is much more likely to be delayed or missed. Certain conditions have many signs and symptoms in common with tuberculosis and must be considered in differential diagnosis. These include pneumonia, bronchiectasis, pulmonary abscess, neoplasms of the lung, pulmonary fibrosis, the pneumoconiosis, and the more recent virus infections of the upper respiratory tract.

Three well-known and time-tested diagnostic aids are available to assist in establishing the diagnosis of tuberculosis. These include the tuberculin test, the x-ray, and the detection of tubercle bacilli in the sputum and body fluids.

Of the diagnostic aids available for assistance in establishing the diagnosis of tuberculosis, we, in this paper, are only interested in the determination of the presence of tubercle bacilli by laboratory methods. The laboratory diagnosis of tuberculous infection involves only the methods that have been developed for the identification of the tubercle bacillus. There is also an increasing use of the laboratory facilities for determining the infectivity of early and convalescent cases. In early tuberculosis the finding of the tubercle bacilli is the most important and sometimes the only decisive finding in the examination of the patient. The aid in diagnosis which the laboratory can furnish by demonstrating the presence of tubercle bacilli in sputa and body fluids is too generally recognized to require emphasis. The most important method of laboratory diagnosis in tuberculosis is the finding of the tubercle bacilli in a suspected fluid or tissue.

There are three methods available for the examination of specimens submitted to the laboratory for tuberculosis. They are: (1) microscopic examination, (2) cultural methods, and (3) animal inoculation. The comparative study presented here deals only with the microscopic examination and animal inoculation.

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The microscopic examination is the original method of demonstrating acid-fast bacilli, and even though it has been refined through concentration methods and is used routinely in most laboratories it still has its limitations.

The tubercle bacilli do not stain easily with the ordinary aniline dyes and belong to a group of bacteria which is known as "acid-fast." These organisms because of a wax contained in them, stain with difficulty, but once stained the dye is retained in spite of treatment with alcohol and strong acids. This acid-fast phenomenon has furnished laboratory workers with a basis for differential staining. However, microscopic examination alone cannot distinguish between tubercle bacilli and other types of acid-fast bacilli, of which there are many. Ziehl-Neelsen's method of staining smears for tubercle bacilli is most generally used in all laboratories.

The direct smear examination is a rough method of screening out the obvious positives. It has been estimated that in order to find acid-fast organisms on a slide there must be 100,000 organisms per c.c. Also one has to consider errors of the technique employed in preparation of smears to be examined. However, despite the limitations of the direct smear, it is universally used as a satisfactory means of examining routine laboratory specimens for evidence of the tubercle bacilli.

The cultural method will not be discussed here because we have been more or less unsuccessful, although more effort is being expended toward improvement of technique, etc., which we hope will lead to better results.

During the past few years several culture media have been devised which seem to give practical results of high efficiency. In cultural work great care should be taken in the collection of the specimens, especially gastric washings and urine specimens; otherwise, the laboratory worker is beset with contaminations. It is hoped that in the near future a comparative study will be presented between cultural and animal methods for the detection of tubercle bacilli.

Animal inoculation continues to be the method of choice in our laboratories. It offers a reliable means for detecting the presence of tubercle bacilli in body fluids, especially those where only a few organisms are found. The guinea pig is the most popular animal and has been used extensively in the past because one can distinguish between pathogenic and nonpathogenic acid-fast bacilli. So far no medium has ever entirely equalled guinea pig inoculation in reliability. The animal inoculation method is time-consuming (at least six weeks), costly, and a certain percentage of the animals die before the end of the test period from secondary infections. The latter factor seems to be the most serious objection to the use of animals. However, as shown in this study, by concentration and digestion of the specimen to be examined and careful handling of the animals, only a small percentage of the guinea pigs injected is lost. Our experience has been unlike that of most laboratory workers in this respect; many reports have shown a loss of from 10 to 20 per cent of inoculated animals from intercurrent infection, but, as shown later, our loss in this series has been only 2.5 per cent.

#### SPECIMENS

The specimens used in this study were submitted by physicians in North Dakota and in all instances animal inoculation was requested. However, the



specimens were examined microscopically as well as by guinea pig injection. The majority of the specimens was received in sterile containers sent out from the laboratory; those not shipped in our containers were sent in in almost any type of miscellaneous container. At no time was any of the specimens more than forty-eight hours in transit, and they were examined microscopically and injected into animals the day they were received.

All specimens of sputa, pleural fluid, pus and gastric washings were concentrated and digested before being examined. Specimens of spinal fluid, hydrocele fluid, and urine were centrifuged in a high speed angle centrifuge for thirty minutes, after which the supernatant fluid was poured off and the sediment used for examination.

#### CONCENTRATION

As noted above, all specimens except urine and spinal fluids were subjected to concentration and digestion. Before examination, each specimen was treated as follows: Equal parts of the specimen and 4 per cent aqueous solution of sodium hydroxide were placed in a wide mouth stoppered bottle. The mixture was then shaken vigorously until homogeneous, placed in a 37° C. incubator for thirty minutes, and shaken at least three times during the incubation period. The specimen was then centrifuged at high speed (approximately 2,500 revolutions per minute) for thirty minutes, after which the supernatant fluid was poured off. A few drops of an indicator (0.04 per cent brom thymol blue) were added and mixed. The mixture was then neutralized by adding drop by drop one per cent HCl. As soon as specimen had been neutralized, it was ready for examination.

TABLE I

TOTAL SPECIMENS EXAMINED MICROSCOPICALLY FOR TUBERCLE BACILLI

SPECIMEN	POSITIVE	NEGATIVE	TOTAL	PER CENT POSITIVE
Sputa	10	86	96	10.4
Pleural Fluid	4	134	138	2.9
Spinal Fluid	1	42	43	2.3
Urine	13	161	174	7.4
Gastric Washings	8	96	104	7.7
Miscellaneous	--	35	35	--
Total	36	554	590	6.1

#### MICROSCOPIC EXAMINATION

Smears were made from the sediment after digestion and concentration of the specimen. New slides were always used in making preparations. Fairly thick smears were made in most instances where there was sufficient sediment. Smears were prepared in the usual manner and stained by Ziehl-Neelsen's method. All smears were examined under the oil immersion lens of a binocular microscope, with wide field oculars, an average of fifteen minutes being spent on each slide.

Table I shows the result of the microscopic examination on 590 specimens. It will be noted that in 36 instances acid-fast bacilli were found; this was 6.1

per cent of the total number of examinations. It is interesting to note that the highest percentage (10.4 per cent) of positives was found in the sputum specimens and the lowest percentage (2.3 per cent), in spinal fluid, although in the miscellaneous specimens (pus, hydrocele fluid, etc.) no acid-fast bacilli were observed in the 35 cases.

#### ANIMAL INOCULATION

After smears were made from the sediment, approximately 2 c.c. of sterile normal saline were added to the remaining sediment; this was shaken vigorously and then the total amount was used for inoculation. Each animal was injected subcutaneously in the inguinal region. All animals were placed in individual cages for a test period of not more than six weeks. (Note later under "Care of Animals.")

TABLE II

TOTAL SPECIMENS EXAMINED FOR TUBERCLE BACILLI BY ANIMAL INOCULATION

SPECIMEN	POSITIVE	NEGATIVE	UNSATISFACTORY	TOTAL	PER CENT POSITIVE	PER CENT UNSATISFACTORY
Sputa	15	80	1	96	15.6	1.0
Pleural Fluid	43	93	2	138	31.1	1.4
Spinal Fluid	14	29	-	43	32.5	--
Urine	22	146	6	174	12.6	3.4
Gastric Washings	30	69	5	104	28.8	4.8
Miscellaneous	5	29	1	35	14.2	2.8
Total	129	446	15*	590	21.8	2.5

\*Animals died before the usual six weeks' test period; reported as unsatisfactory; microscopic examination negative in all instances.

Table II shows the results of the animal inoculation on the same 590 specimens as listed in Table I for microscopic examination. A total of 129 cases, or 21.8 per cent of the guinea pigs, were found to be positive. The highest percentage (32.5 per cent) of positives was obtained with spinal fluid specimens, and the lowest (12.6 per cent), with urine specimens. In 15 instances, or 2.5 per cent of the total number of animals inoculated, results were unsatisfactory. In all of these cases the animals died from one to three weeks after injection. All animals were autopsied and death accounted for from intercurrent infection. Microscopic findings were negative in all 15 specimens used for inoculation. Premature mortality in only 2.5 per cent of the total cases does not seem to be a very high incidence of unsatisfactory cases.

Table III shows the results obtained on the specimens by both the microscopic examination and animal inoculation. As noted, the total number of positives obtained by both methods was 142, or 24.0 per cent of the total. By the microscopic examination, 36 cases, or 6.1 per cent, were positive, and by animal inoculation, 129 cases, or 21.8 per cent, were positive.

This means that by animal inoculation we were able to pick up 93 more cases (15.7 per cent) than by microscopic examination or a combination of the two methods. One can readily see that those fluids which are exceedingly difficult to examine microscopically gave a significantly higher number of positives through animal injection; i.e., on pleural fluids we had 43 positives with inoculation and only 4 positives microscopically; on spinal fluid, 14 positives

with inoculation and 1 positive microscopically; on gastric washings, 30 positives with inoculation and only 8 positives microscopically.

## COMPARISON OF METHODS

Table IV shows complete agreement and disagreement between the two methods on the specimens. It will be noted that in only 23 instances was there complete agreement between the two methods as far as positive results are concerned. This is only 4 per cent of the total number of specimens examined. There was complete negative agreement in 433, or 75.3 per cent, of the cases. Interesting to note is the fact that in 106 cases, or 18.4 per cent, there was complete disagreement; the microscopic examination was negative and the guinea pig, positive. This is the most significant fact shown by this study. In only 13 cases, or 2.3 per cent, were microscopic findings positive and the guinea pig negative. These 13 cases were accounted for as follows:

TABLE III

TOTAL SPECIMENS EXAMINED MICROSCOPICALLY AND BY ANIMAL INOCULATION FOR TUBERCLE BACILLI

SPECIMEN	TOTAL NO	TOTAL POSITIVE ANY METHOD	PER CENT POSITIVE	ANIMAL		MICROSCOPIC	
				POSITIVE	PER CENT POSITIVE	POSITIVE	PER CENT POSITIVE
Sputa	96	20	20.8	15	15.6	10	10.4
Pleural Fluid	138	44	31.8	43	31.1	4	2.9
Spinal Fluid	43	14	32.5	14	32.5	1	2.3
Urine	174	27	15.5	22	12.6	13	7.4
Gastric Washings	104	32	30.7	30	28.8	8	7.7
Miscellaneous	35	5	14.2	5	14.2	--	--
Total	590	142	24.0	129	21.8	36	6.1

TABLE IV

COMPARISON OF MICROSCOPIC EXAMINATION AND ANIMAL TEST ON SPECIMENS FOR TUBERCULOSIS

EXAMINATION	TYPE OF SPECIMEN									
	SPUTA		URINE		PLEURAL FLUID		GASTRIC WASH.		MISCELL-ANEOUS	
	NO	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
Microscopic Guinea Pig										
Complete Agreement	75	78.9	141	84.0	92	67.6	67	67.7	58	75.4
Negative-Negative	5	5.3	8	4.7	3	2.2	6	6.1	1	1.2
Positive-Positive										
				88.7	95	69.8	73	73.8	59	76.6
				8.3	40	29.5	24	24.2	18	23.4
				3.0	1	0.7	2	2.0		13
Total	15	15.8	19	11.3	41	30.2	26	26.2	18	23.4
Grand Total	95		168		136		99		77	

\*Fifteen animals died before completion of test period (not included in total).

There were 5 urine specimens, 5 sputum specimens, 2 gastric washings, and 1 pleural fluid. The urines were not catheterized specimens, and one has to consider that the acid-fast organisms observed microscopically could have been nonpathogenic. In the other specimens the organisms could have been rendered unviable during digestion and concentration, or they may have died in transit. No attempt is being made here to give an explanation of why these cases were missed through animal inoculation for no definite proof could be

obtained, inasmuch as all specimens had been discarded before the animals were killed. We have, however, mentioned several factors which could have been involved.

#### CARE OF ANIMALS

The proper care of animals used for isolation of tubercle bacilli is important. The guinea pigs used in this study were of no particular strain; only healthy animals weighing between 250 to 300 Gm. were injected. Each animal was placed in an individual cage with a removable tray at the bottom which tends to keep the cage clean at all times. The animal room is warm and bright with sunshine approximately five hours a day. Each animal is fed and watered every morning, the usual food being "Purina Complete Ration Checkers," supplemented with hay, carrots, and greens when obtainable. Each cage is clearly marked as to date of inoculation and date due for autopsy: at the completion of each test period the animal cages are thoroughly cleaned and sterilized before a new animal is placed in them. At the end of the six weeks' test period, if the animal has not died previously, it is autopsied and examined for typical tuberculosis findings. Smears are generally made to confirm diagnosis except in the most frank cases.

#### CONCLUSIONS

1. In the routine examination of specimens for evidence of tubercle bacilli, microscopic examination should be supplemented by animal inoculation.
2. Specimens for detection of tubercle bacilli should be examined routinely by at least two methods.
3. In the 590 cases examined for the detection of tubercle bacilli, animal inoculation proved to be far superior to the microscopic examination.
4. A certain percentage of "positives" will be missed by any type of examination when a comparative study is made.

## AN ADHESIVE NONDRYING ELECTRODE PASTE\*

WILLIAM J. TURNER, M.D., AND CHARLES S. ROBERTS, M.A., NORTHPORT, N. Y.

A TIME-CONSUMING measure in electroencephalography, as well as a frequent source of artifact, is the application of electrodes to the scalp. Gibbs and Gibbs<sup>1</sup> state that "no one should consider himself trained in this procedure until he has applied at least 300 electrodes. An expert will not have to replace one electrode in 50." During a recent course which was conducted at this Facility, it became imperative to enable an inexperienced person to apply electrodes with ease and assurance. It seemed to us that an improved paste might be of value in this regard.

We thought a desirable paste should be nondrying and adhesive. Although perfect adhesiveness has not yet been achieved, we have settled on the following formula for a tacky paste which does retain its moisture indefinitely:

Bentonite, 200 mesh, 100 Gm.  
Saturated solution of calcium chloride,  
85 c.c.

This is simply mixed into a paste and transferred to a suitable container. We have kept this paste in an open jar for several months and found that only a few drops of water were then required in order that it regain maximum stickiness. We apply a thin layer to an electrode which has been corrugated on one surface. (We prepare our electrodes by melting a small ball of rosin-core solder about the coiled end of a fine copper wire, and by pressing on the molten ball firmly and quickly with a nail file.) The electrode is applied in the usual manner. Where the hair is too short, the electrode is held in contact with the scalp by a hard wax pencil. A few drops of 3 per cent collodion in acetone-ether are applied over the electrode and dried rapidly by an air blower.

With this paste a wholly inexperienced person is able to apply 8 electrodes in fifteen minutes with complete assurance. Approximately 160 electroencephalograph examinations have been performed, using this paste, without the occurrence of a single electrode artifact. Comparisons with the usual electrode paste tracings reveal no significant differences in the record. Electrodes have been left on for hours without evidence of drying out of the paste.

### REFERENCE

1. Gibbs, F. A., and Gibbs, E. L.: Atlas of Electroencephalography, Cambridge, Mass., 1941, Lew A. Cummings Co.

\*From the Neuropsychiatric Research Unit, Veterans Administration Facility.  
Research Paper No. 6, Neuropsychiatric Research Unit, Veterans Administration, Northport, New York. Published with the permission of the Medical Director of the Veterans Administration who assumes no responsibility for the views expressed herein.  
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## DARK-FIELD DIAGNOSIS OF PENILE LESIONS\*

### DIFFERENTIAL MOTILITY CHARACTERISTICS OF *TREPONEMA PALLIDUM*

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THE earliest possible diagnosis of syphilis by the several laboratory procedures invariably points to a first choice of the dark-field procedure. Even with the advent of the more sensitive serologic tests, comparative data disclose the highest percentage of positives are obtained with dark-field examination. McNabb, Matthews, and McClure<sup>1</sup> found 72 per cent positive by dark field compared with 64 per cent by multiple sensitive flocculation tests, with stained smear diagnosis ranking third with 59 per cent positive. In seronegative cases they found 94 per cent positives by dark field.

Corollary tests or procedures to supplement the dark-field method, because of unfamiliarity with its technique, have been suggested. McDaniels<sup>2</sup> considered the value of serologic study of fluid aspirated from lesions but admitted the difficulties attendant on consistently obtaining suitable material. In a comparative study of dark-field examinations with the nigrosin stain, Nagle and Graul<sup>3</sup> found seemingly close correlation. Here again variable factors inherent in the study of smears obtain, such as distortion by drying, pseudospiral organic matter, etc.; these are common to all types of stain preparations. In this connection, we are in agreement with Casselman<sup>4</sup> that none of the various staining methods, silver impregnations, aniline dyes, or contrast backgrounds of the India Ink type should supplant the dark-field test. No other procedure can yield such itemized criteria as does dark-field study of the living syphilis organism. What difficulties and errors in diagnosis attend dark-field studies can be laid at the door of unfamiliarity with characteristic motility and morphology. Stokes<sup>5</sup> has rightly questioned the desirability of placing dark-field apparatus in the hands of the untrained or inexperienced laboratory worker. As he states, "after seeing some of what purports to be *treponema pallidum* in current moving pictures, one begins to wonder whether the only real one is that observed by darkfield from lymph nodes and from scraped papules on the glabrous skin in undoubted secondary and recurrent eruption." It is our purpose to describe in fullest detail the dark-field features of *Treponema pallidum*. Sustained observation of the spirochetes has disclosed a very broad range of motility characteristics. Available texts do not describe these in sufficient detail.

#### MATERIAL AND METHOD

We have had the opportunity of studying early lesions in a military hospital where optimum conditions for sustained case studies existed. Repeat and follow-up checks were complied with uniformly. Kahn and Wassermann tests and smears stained by a wide range of indicated stains were routinely made. It was always kept in mind, however, that these procedures were never more than corroborative.

\*From the Station Hospital, Camp Cooke, California.  
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Many of the penile lesions were even too early to be suitable for dark-field examination. Patients have reported to the laboratory within one to three days following the appearance of minor sores or "pimples" of 1 to 2 mm. diameter. The first appreciable serum obtainable after daily attempts presumably contained the earliest organisms with the attendant vigor of a young growth. Motility here would be at its optimum for study. Inhibition of the several forms of motility would be at its minimum. With such highly suitable material, the highest possible efficiency in dark-field diagnosis is reached, i.e., well within the first two weeks of the primary lesion.

One hundred consecutive cases of penile lesions were examined in a six months' period, 14 of which were positive for syphilitic spirochaeta. Dark-field search was made on all lesions and repeated daily or on alternate days for a series of four examinations on the individual patient before reporting negatives. The interval of one or two days was based on the appearance and size of the lesion. Those of greater extent were observed over six to eight days. In a few cases, small nonspecific lesions or superficial ulcerations healed within two or three days, terminating further laboratory examinations. None of these latter sores were ever found positive for the syphilis organism.

Several cautions in dark-field procedure were constantly observed to reduce the fallibility of various factors:

1. *The Location, Character, and Size of the Lesion (or Lesions).*—These were studied for suggestive clinical features. It cannot be emphasized too strongly that the lesion may or may not conform to the classical description of the hard chancre. Eisendrath and Rolnick<sup>6</sup> list seven forms of syphilitic chancre. They point out how often any apparently simple lesion proves to be a primary one. We are in accord with Brown<sup>7</sup> and, more particularly, Branch,<sup>8</sup> who have stressed the frequent absence of typical features of the syphilitic chancre—the hardness, painlessness, and swelling of inguinal nodes. In a high proportion of cases we have found lesions ranging from shallow, soft minimal ulcerations (quasi abrasions) to multiple broad necrotic sloughs, in all of which the presence of painlessness or induration was disconcertingly inconstant. Many lesions were so fully infected, covered with pus or tenacious shaggy fibrin, as to render collection of serum difficult. We observed two lesions which were essentially deep-seated abscesses with erosion and rupture through small skin perforations, from which considerable inspissated pus could be expressed. Surprisingly, one of these was positive for *Treponema pallidum* with a simultaneous 3 plus Kahn. That this secondary suppuration is sufficiently frequent and can interfere with dark-field examination is attested to by various attempts to obviate this difficulty. Friedman<sup>9</sup> has recently devised a method for proper preparation of this type lesion following procedures suggested by earlier authors.

Two lesions consisted of nothing more than a diffusely hyperemic, moist glans penis with apparent denudation of large areas and no recognizable focal ulceration. A free flow of serum was positive in both cases. A case of erosive balanitis due to Vincent's infection was encountered, of the type discussed by Madden.<sup>10</sup> It is desirable but not essential to remove the interfering pus cells from the serum. The feature of interfering bleeding and blood contamination again was observed to bear no constant relation to the character of the lesion.

The older, characteristic, true chancre withstood cleansing manipulation more readily without appreciable bleeding but this was by no means invariable.

2. *Efficiency of Collection of Material.*—Two much care cannot be exercised to insure, first, a clear serum. Secondly, as copious an amount (or flow) of serum as possible should be obtained. This is especially important in the early or small lesion or in a nearly healed lesion examined for the first time. Thirdly, serum should be collected by a capillary tube as near as is possible to the depths of the lesion, in preference to fluid obtained from the walls or edges of an existing crater. After the usual sponging and direct cleansing of the lesion with saline-soaked cotton, repeated once or more if bleeding or pus exudes, the lesion is wiped dry and readied for collection of serum. Direct squeezing, expression, or suction bulb manipulations of the lesion were found unsatisfactory, as bleeding occurred too often. The most efficient method was to induce marked venous congestion just proximal to the lesion, maintained continuously for as long as five minutes by an annular grasp by the operator's thumb and forefinger. The constricted end of the penis assumes a dusky red or even cyanotic hue. The stasis of the organ around the chancre usually induces a ready flow of serum. The serum may flow copiously even after the release of constriction. Bleeding is minimized since no direct trauma or drastic suction is exerted on the raw, exposed surface. Little or no tissue particles, or pus or fibrin, usually liberated by direct squeezing, will be mixed with the serum.

3. *Efficiency of Dark-field Examination.*—(a) *Equipment.*—The several makes of dark-field apparatus available are all efficient. There is some advantage to those equipped with a substage 6 to 8 volt lamp. In addition to the optically fixed light source the moderate amount of heat generated warms the slides sufficiently to enhance or maintain motility of microorganisms.

(b) *Darkness of Field and Amount of Fluid.*—The thickness of the fluid layer examined will affect the darkness of the field. The thicker the layer, the blacker the background. Undue pressure as by raising the substage condenser too much will tend to render the fluid dark-brown, lowering contrast of the illuminated suspended objects. In addition, too much fluid causes disconcerting currents when focusing, because if the coverslip "floats," vertical motion of the objective in focusing will depress or release the coverslip. As the fluid dries out or changes temperature, convection currents arise and a too rapid flow of fluid across the oil immersion field occurs. The organisms studied will have to be "chased after" by moving the mechanical stage too rapidly. The serum drop should just reach to the margins of the coverslip. Drying may be retarded by vaseline—ringing the edges if prolonged observation is also needed. An overflow along the coverslip edges with too much serum will cause these rapid currents. Too large a drop with a greater depth of the fluid allows the organism to move vertically or "dive" too quickly out of focus. The spirochete will be viewed end on instead of laterally; coil arrangement and motility are rendered difficult to determine. At the other extreme, too small a drop in the center of the coverslip preparation gives too shallow a fluid layer, which, drying rapidly, cuts short the useful period of dark-field observation.

(c) *Interfering Factors.*—Pus cells, glass scratches, and red cells generally are so highly refractile that a blinding glare results. Visual accommodation for spirochetal outlines is rendered difficult. The effect is comparable to the situa-



tion in driving a car at night. One knows how hard it is to concentrate on dimmer objects along a roadside in the same field of view as an oncoming car's blinding headlights. The importance of obtaining as clear a serum as possible is readily understood.

(d) *Artefacts Simulating Spirochetes*.—Glass scratches even on new slides or coverslips of good quality will occasionally have a quasi-spiral appearance. Their obvious nonmotility and inconstancy in size rules them out quickly. Bacterial forms, such as a granular more or less bent rod, will impart a false spiral outline. A short streptococcus chain will give a row of refractile points. With active Brownian movement and possibly an annoying current, definitive focusing may be difficult to maintain. Errors could arise in this manner.

Our findings in 100 patients with penile lesion may be tabulated as follows:

TABLE I  
PENILE LESIONS POSITIVE FOR SPIROCHETES

CASE NO.	TR. PALLIDUM	B. REFRINGENS	SP. PHAGEDENIS	VINCENTS' SPIROCHETES
1	1			
2	1			
3	1			
4	1			
5	1			
6	1			
7	1			
8	1			
9	1			
10	1			
11*	1			
12*	1			
13	1	1		
14	1	1		
15		1		
16		1		
17		1		
18		1	1	1
19			1	
20			1†	
Total	14	6	3	1

\*Double or "Kissing" lesions, both positive for *Tr. pallidum*.

†Possible microdentium, found in one of five daily examinations on the same case.

Brief mention of the several spirochetes may be made. As seen in this tabulation, infection with two species occurred three times, twice with the pallidum, once with two nonspecific spirochetes. One small delicate organism listed as a possible microdentium was seen; follow-up clinical and serologic studies ruled out syphilis in this case. Branch<sup>8</sup> found a ratio of 40 syphilitic spirochetes to 11 nonspecific spirochetes. Our ratio of 14 pallida to 10 nonspecific organisms is higher. Our series is too small to draw statistical comparison.

#### MOTILITY CHARACTERISTICS OF *TR. PALLIDUM*

In textual descriptions of the several spirochaeta, physical characteristics of movement and shape have been loosely combined. They can be separated as they can be demonstrated to be independent of one another. Locomotion or progression is one form. The other form is related to changes in shape or outline.

The following discussion is concerned chiefly with the specific spirochete of syphilis. It is felt that thorough familiarity with the physical behavior of this

organisms will facilitate differentiation from other genital spirochaeta. Since differential tabulations of several nonspecific species are available in the detailed review by Rosebury<sup>11</sup> and elsewhere,<sup>12 a, b</sup> only pertinent reference to distinctions among penile spirochaetae will be made.

Consideration of the physical behavior of the spirochetes will be greatly clarified by comparison with that of a steel coiled spring of a quality and elasticity encountered in precision instruments. This example instead of the "corkscrew" description is preferable. A spring can be changed in shape, not so a corkscrew. Keeping in mind the coil spring structure, the side illumination of the dark-field does not light up uniformly each loop of the spiral. The light generally seems to strike on the front or nearer lateral half of coils. The effect is very often a series of bright commas or parenthesis marks. The usual description of a "series of dots" is less accurate. Constant focusing is necessary to delineate the faint posterior semicircles connecting these commas. Stitt<sup>13</sup> points out that this "beaded" effect is seen when the organism is at rest, the spiral structure revealed only when it is in motion. A nonmotile spirochete could be missed on this basis.

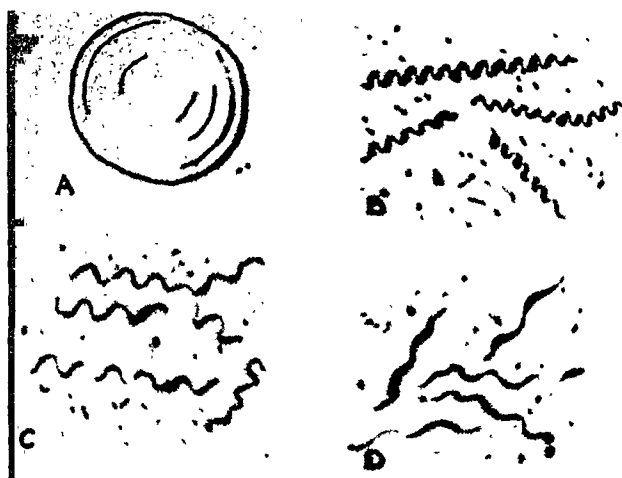


Fig. 1.—Common penile spirochaetes in the dark field. A, red blood cells; B, *Treponema pallidum*; C, *Borrelia refringens*; D, *Spirochaeta phagedenis*.

#### MOTILITY FORMS OF *TREPONEMA PALLIDUM*

A. *Motility of Locomotion*.—True motility is herewith defined as any motion which changes the spatial relation of the organism to a fixed point in the field of view, i.e., any swimming or traveling motion. There are two types of locomotion:

1. *Rotation*.—This is moderately rapid, on an imaginary longitudinal axis of the spiral and clockwise or counterclockwise. Maximum speed or rate of rotation of pallidum never reaches the velocity of the refringens or phagedenis. Rotation may be sluggish, or leisurely. It may be absent in a dying organism or in lesions after one or two days of antiluetic systemic or local therapy. It is of course most rapid in early lesions.

2. *Propulsion*.—This implies forward or backward axial motility of the

length of the spiral and has been termed "translation" by Rosebury. Other terms are: progression, forward (or backward) movement. In the case of *Treponema pallidum*, the motion of active rotation, usually predominant, may give an illusion of exaggerated propulsion. Generally speaking, little removal of the spiral from one spatial point to another occurs. If it does, it is unusually slow, intermittent, and just as often reversible. (Fluid currents carrying the organism out of the field will give a false impression of propulsion.) A rapid or slow forward or backward "darting" movement is best seen in the refringens or phagedenis organisms, sometimes necessitating chasing the organism from one field to another. This will never be the case with pallidum. The queen of spirochetes has appropriately only a stately and unhurried travel, is never fitful or spasmodic in its motion.

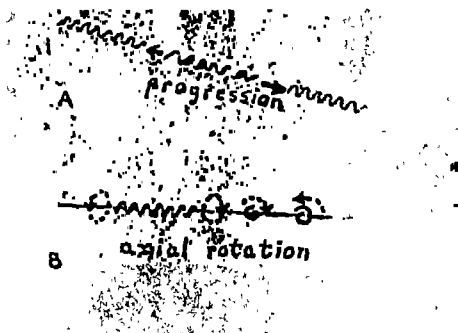


Fig. 2.—*Treponema pallidum*—motility of locomotion. A, progression; B, rotation. (See text.)

**B. Motility of Change in Shape.**—Prolonged observation of the several spirochetes impresses one strongly that the *Spirochaeta pallida* has almost unlimited scope in changing its outline. None of the other penile spirochetes has the protean forms in the literal sense of the term "protean." These changes in form can be listed as (1) flexion or angulation, (2) "buckling" or distortion of its coils, (3) undulation or fluctuation of the coils, (4) compression of its coils, (5) expansion of its coils, (6) looping. In general, the subtypes of movement in Group A are far more prominent in the nonspecific organisms, while the variations in Group B are more evident in the syphilitic organism.

1. *Flexion or Angulation:* Of the six types of shape listed, if one had to designate any diagnostic motion of the pallida, flexion or angulation could be so indicated. This is a constant feature and is present in from 10 to 30 per cent of all moving pallida seen. No other spiral form in the dark-field shows the degree and incidence of this motion.

The bending or angulation is usually obtuse, but can be acute. The point of rotation may be anywhere along the length, usually near the junction of thirds. The longer arm is stationary, while the shorter arm sways and swings leisurely occasionally assuming momentarily a V shape. The fulcrum of angulation is possibly the point of transverse division in reproduction. Although

physical division into two organisms has been searched for diligently in vigorous organisms from early, highly infected lesions, only once have we seen it, after prolonged observation.

2. *Buckling*: This is the kind of shape that reminds one most typically of a coil spring being forcibly compressed by end pressure. Some central coils will resist this action and spring out of line. The spring is said to buckle. This is precisely what seems to occur to the *Spirochaeta pallida*. Buckling is almost as frequent a behavior as angulation. It is most characteristic of the pallida, but it can be fleetingly observed in the refringens spiral. Distinction is easy, as the buckled coils in the pallida will remain for seconds at a time.

3. *Undulation*: Synonymous terms used by various authors are quivering, vibration, fluctuation, (inaccurately) peristalsis. If one bears in mind the semirigid character of a coil spring, which can "quiver" if tapped lightly, the behavior of the *Spirochaeta pallida* is better understood. The long axis of the organism undulates or fluctuates about a central fulcrum in a manner similar to that of a magnetic compass needle on its center bearing. This motion is more apparent with the organism in the stage of rest, i.e., when no rotation or angulation is present. The nonspecific organisms have similar undulatory movements, but there is more suppleness or "spineless" flexibility to the axial body, a good differential point.



Fig. 3.—*Treponema pallidum*—motility of change in shape. A, angulation; B, buckling; C, coil compression; D, coil expansion; E, looping; F, undulation. (See text.)

4. *Compression of the Coils*: This action and its corollary, expansion, are mentioned in but few of the standard texts. Compression of the coils occurs noticeably frequently in the active pallidum, is usually fleeting, and may be partial, involving one segment of the organisms. The refringens can also compress its coils, but at a less frequent rate or not at all. In the pallida the length between coils will be correspondingly narrower (less than 1 micron). The coils may not be regularly spaced as in the resting or rotating phase. It is this motion of compression that is commonly called peristalsis or undulation.

5. *Expansion of the Coils*: This action is independent of the preceding one. The coils are stretched out from a resting state. Compression may or may

not have preceded this opposite movement. Usually the coils stay stretched for a longer period, up to several seconds (cf. the split-second duration of the compression phase) before a literal "recoil." The increase in length of the organism is approximately one-fourth. The refringens and phagedenis organisms behave in a similar manner. This action thus cannot be used as a differential point, since the frequency of coil expansion is about the same in all the organisms, although easily discerned in the larger refringens. The terminal pointed filament of the *Spirochacta pallida* as usually described has been infrequently demonstrated in our dark-field studies. However, when present, they become more distinct and longer in the pallidum in the phase of expansion.

6. *Looping*: In highly motile pallida, especially in the first few days of a lesion, looping of the axis to form an ellipse or narrow-throated U is not infrequent. One pole of the pallida remains fixed; the other pole arcs slowly to approximate or meet in a closed or open loop. The loop may remain closed for from seconds to a minute. While in this state the whole loop may slowly rotate on a transverse diameter, like a ring transfixed on a rotating pole. It may give the appearance of a "tumbling" or end-over-end movement.

In one 3-day-old lesion with a profusion of organisms in the dark field this looping shape was modified peculiarly. The ends would cross to form for seconds at a time a script letter "e" or "l" or even a Greek script letter "α." While in this position the loop occasionally closed completely to form a script "i." This was the only instance in which the characteristic rigidity or linear pattern of a coil spring was not maintained. Five per cent of the organisms in this same case showed a wavy motion of the coils to form constantly varying S-shapes. This bizarre behavior introduced doubt as to the specificity of the organism. The clinical features, the lesion, and the appearance of repeated positive Kahns and Wassermanns three weeks later was sufficient corroboration.

We have not observed looping and its allied motions as described here in the nonspecific spirochetes. A simulated looping or blunt V formation has been seen to occur in highly active refringens without the complex variations of the looping movements in the pallida. Diminution of motility as in lesions of over two weeks, or after treatment, obviously will reduce the variety of motions. The few sera from secondary closed lesions that we have studied disclosed little more than rotation. Agee<sup>14</sup> believes that the lessened activity of the organisms is caused by pressure in obtaining serum. Our procedure for obtaining fluid by venous stasis precludes this mechanical effect. There is no reason why the decline in numbers of organisms, as antibody response in late primary and secondary stages rises, is not coupled with lessened motility.

With the above numerous extrinsic and intrinsic types of motility in mind, it is quite evident that the pallida has versatility and protean faculties far above and beyond any other spirochetel forms that may be encountered. A point that must be re-emphasized in any consideration of its differential diagnosis is the relatively moderate or even slow rate of motility in any of the types listed above. The gentle or stately or leisurely waving or bending in the pallida is always unhurried. The behavior of seaweed in the surf, or that of pliant fields of grain in a breeze is analogous in its grace and undulations to the physical motion of the pallid spirochete.

It is hoped that familiarity with the characteristic details of motility will

obviate many errors in dark-field diagnosis of *Treponema pallidum*. Uncertainty that makes one seek the secondary corroborative procedures of serologic tests, stained smear studies, or doubts of clinical impression should be minimized by the procedure of the tried and true dark-field examination of suspected syphilitic lesions.

## SUMMARY

1. The diagnosis of early syphilis in penile lesions is best determined by dark-field study. Other laboratory methods should be used for corroboration.

2. A number of cautions are outlined which will minimize artifact errors and pitfalls in the dark-field study of penile spirochetes. The source material and lesions are so often atypical that any penile lesion or lesions may reveal *Treponema pallidum* by dark-field examination.

3. The *Treponema pallidum* has a range of motility characteristics so broad that it should make for little difficulty in differential diagnosis.

4. Motility of penile spirochetes relating to locomotion is distinguishable from motility relating to change in shape. Eight types of motility of *Treponema pallidum* and other spirochetes are described under two main groups. Several of these are so characteristic to be of pathognostic value in the diagnosis of syphilis by the dark-field method.

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## A METHOD FOR MOUNTING GROSS PATHOLOGIC SPECIMENS IN PETRI DISHES\*

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THE customary method for preserving and mounting gross pathologic specimens by the use of the museum jar is somewhat difficult and time-consuming. The jars are usually heavy, cumbersome, and are fairly expensive; and a considerable amount of space is required to store or display them. These disadvantages preclude the use of jars in many hospitals.

In those cases where the specimen is small or where a cross section suffices to show the gross pathologic features, it is possible to dispense with the use of jars by utilizing a method developed by Watters,<sup>1, 3</sup> Coplin,<sup>2</sup> Day,<sup>4</sup> Judah,<sup>5</sup> Warren,<sup>6</sup> Davis,<sup>7</sup> Larson and Levin,<sup>8, 10</sup> and Bengston.<sup>9</sup> The specimen is mounted in half a Petri dish or watch glass which is filled with fluid and sealed to a glass plate. Mallory<sup>11</sup> states: "The method has the advantages that the materials used are cheap and the space needed for the storage of finished specimens is small. It has the disadvantage that it is somewhat time-consuming and difficult technically for the beginner."

In the method presented in this paper, the whole Petri dish, both bottom and cover, is used, thus dispensing with the somewhat cumbersome glass plate. The present method is rapid, technically simple, and even more space-conserving than the mounts of Petri dish and glass plate. Furthermore, the mounted specimens are convenient to handle and examine and can be readily photographed.

*Method.*—Two sizes of Petri dishes have been found suitable for most specimens. These are the 100 by 15 mm. and the 150 by 20 mm. In addition, crystallizing dishes, 90 by 40 mm. and 145 by 60 mm., covered with the tops of Petri dishes, have been used for some specimens. Very large Petri dishes, 9¼ by 1¼, 11 by 1¼, and 13 by 1¼ inches, are available for cross sections of large organs like the whole lung or for coronal sections of the brain.

The gross specimen, preserved in formaldehyde or Kausler's solution, is trimmed and oriented in the dish. In an occasional case, it is necessary to use some means to prevent the movement of the specimen. One method is to sew the specimen on a sheet of transparent film which is cut to fit the bottom of the dish. This method was used for mounting a surgical specimen of a carcinoma of the common bile duct (Fig. 1). A more rapid method is the use of thin strips of adhesive plaster to attach the specimen to the bottom of the Petri dish. It has been found that if both ends of the adhesive plaster are attached firmly to the dry glass, there is little danger of the strips becoming loose when the fluid is added. An example of this method is shown in Fig. 2. Carcinomas

\*From the Tumor Research Unit, Veterans Administration.

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Fig. 1.—Surgical No. 21-127. Carcinoma of common bile duct with metastasis to lymph node. The surgical specimen is sewn on a photographic film. The duodenum and the common bile duct have been opened. A probe is inserted through the terminal portion of the pancreatic duct which opens directly into the duodenum. The terminal 0.5 centimeters of the common bile duct, is diffusely thickened and superficially ulcerated. The smaller specimen is a cross section showing the pancreatic duct surrounded by a zone of fibrotic tissue. A small lymph node in the pancreatic capsule is infiltrated with tumor.



Fig. 2.—Autopsy No. 40-275. Lymphosarcoma of small intestine. The section of jejunum has an annular ulcer. The base is smooth and white in color.



of the larynx are mounted in 90 by 50 mm. crystalline dishes (Fig. 6). Wads of cotton or a section of a sponge are placed underneath the larynx so as to immobilize the specimen and to bring the lesion close to the glass cover. These three mechanical devices are required only in a small percentage of cases. In most cases the specimen is placed free in the dish (Figs. 3 to 5).

The next step is to fill completely the dish with the mounting fluid in such a manner as to avoid air bubbles in the final mount. A large pail is filled with the solution used for mounting. The fluid should be at room temperature or slightly warmer so as to eliminate as much as possible dissolved air. The bottom dish with the specimen is immersed in the solution. Any entrapped air can be readily released by manipulating the specimen. While one hand holds the bottom dish, the other hand introduces the cover sideways into the solution so as to evacuate all the air. Then the cover is placed over the bottom of the Petri dish. In doing this, both parts of the dish are kept continuously and completely submerged. While keeping the dish closed and submerged, it is inverted so that the cover is beneath the bottom of the dish. The inverted dish is then removed from the fluid.



Fig. 3.—Autopsy No. 40-52 Implantation of ureters into sigmoid colon. The autopsy specimen was obtained nineteen months after the operation. The sigmoid has been folded to show both implants; a probe has been inserted in the terminal portion of the left ureter, and both ureters show moderate thickening and fibrosis. At the junction of the left ureter and colon, a papilla, 2 cm. in length and 1 cm. wide, can be seen. There is no papilla at the junction of the right ureter and sigmoid. The opening of this ureter in the sigmoid is markedly constricted.

The next step is the sealing of the dish. White twine is introduced in the space between the bottom and cover. The twine is pushed down by means of a paper clip. It is run around the circumference of the dish two or three times. Plasteline is then packed tightly in the space between the bottom and cover. The dish may now be handled freely. The outside of the dish is washed free of the mounting solution and dried. The free edge of the plasteline is shellacked.

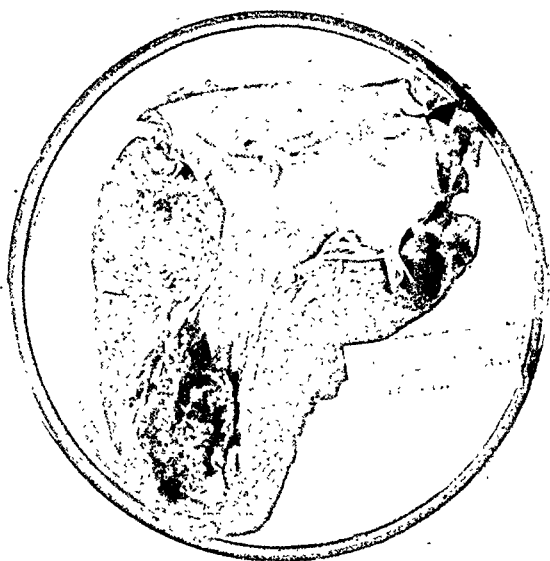


Fig. 4.—Autopsy No. 41-139. Myocardial infarct and mural thrombus. A large, dark-colored thrombus is attached to the apex, the anterior wall, and the interventricular septum of the heart. The myocardium of the anterior wall has a large anemic infarct.

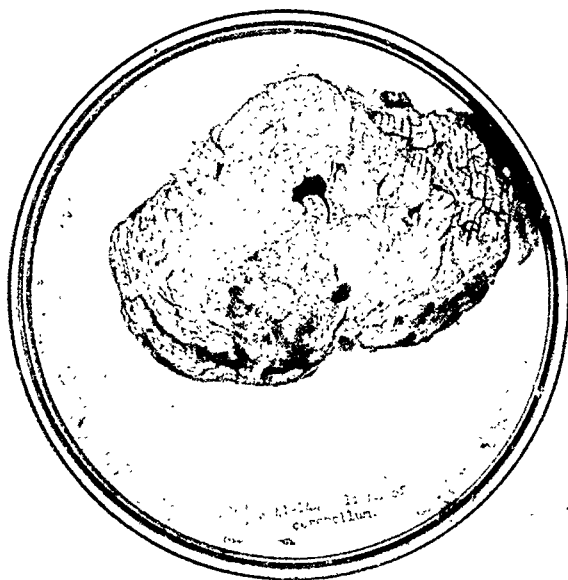


Fig. 5.—Autopsy No. 41-144. Glioma of cerebellum. The right cerebellar hemisphere has a large hemorrhagic, poorly circumscribed tumor which extends to the midline.

A gummed label is attached to the dish. The label is protected by covering it with transparent lantern slide edging.

*Specimens Suitable for Mounting.*—Cross sections of the brain mounted in 150 by 20 mm. dishes are ideal for the demonstration of tumors and other lesions. Hemisections of the kidney can usually be mounted in the 150 by 20 mm. Petri dish. Tumors of the lung can frequently be adequately represented by a cross section which shows the relationship of the tumor to the bronchi and hilum of the lung. A section of the spleen or liver suffices in many cases to show the gross lesion, such as a metastatic tumor, granuloma, or cirrhosis. Ulcers of the gastrointestinal tract can be mounted in the 100 or 150 mm. Petri dish.



Fig. 6.—Surgical No. 18-680. Carcinoma of the larynx. The right vocal cord is destroyed by a large ulcer which infiltrates subglottically and involves both the anterior and posterior commissures. The edges of the ulcer are raised and nodular.

Tumors of the gastrointestinal tract or the genitourinary tract frequently require the 150 by 60 mm. crystalline dishes. Cross sections of the heart suffice to show metastatic tumors. Longitudinal sections are used to show infarcts and mural thrombi. The valves of the heart are sometimes excised and mounted separately. Thin sections of the vertebrae are used to show such lesions as metastatic tumors, Paget's disease, and multiple myeloma.

The specimens shown in Figs. 1 to 5 are all mounted in 150 by 20 mm. Petri dishes. It should be noted that the mounted specimens photograph satisfactorily.

*Storage of Petri Dishes.*—A special cabinet, 60 by 24 by 22 inches, was built to store the Petri and deep dishes. It has 18 flat drawers, 21 by 19 by 1¾ inches, which have movable partitions to accommodate either the 150 by 20 mm. or the 100 by 15 mm. Petri dishes. Each drawer holds 9 of the larger dishes or 3 large and 12 small ones. The cabinet also has 2 deeper drawers, 21 by 19 by 3 inches for the crystallizing dishes. This small cabinet accommodates from 180 to 300 museum specimens, depending upon the size of dishes used.

By use of such a cabinet, a large number of gross specimens can be stored compactly. Furthermore, the dishes are protected from dust and are readily accessible for examination.

#### SUMMARY

A simple method is presented for mounting surgical and autopsy specimens in Petri dishes and the filing and storage of mounted specimens in a cabinet.

The method is easily learned, is rapid, and permits the filing and storage of a large number of mounted specimens in a relatively small cabinet.

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## A SIMPLE AUSCULTATORY TECHNIQUE FOR THE ESTIMATION OF THE BLOOD PRESSURE OF DOGS\*

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SEVERAL auscultatory techniques have been described for the estimation of the blood pressure of dogs.<sup>1-3</sup> They have required special cuffs, racks, and other devices, which, in addition to being cumbersome, offer difficulty of adaptation to dogs of different size. A simple modification of the auscultatory technique is described here. It consists of a method for holding the rubber bag which is to be inflated firmly in place.

*Method.*—A roll of semielastic "Ace" bandage which is used in taping ankles (dimensions: nine feet by two and one-half inches) is stitched to the tail of the cloth cover of a blood pressure cuff (dimensions: four by thirty inches). The cuff is applied to the thigh of the dog, the bandage being wrapped around until every portion of the cuff is covered, a few turns being taken up around the torso during the procedure (see illustration). The diaphragm of a stethoscope is inserted under the lower edge of the cuff over the femoral artery. The blood pressure is then measured in the conventional manner. The sounds are generally fainter than in human beings, and in short-legged, stocky dogs too faint for reliable readings, but in most cases the end points are equally sharp. A few trials will indicate the best position for the cuff and stethoscope in the individual animal; in some cases the sounds are heard more clearly below the knee. If the cuff is too tight, sounds may be heard below diastolic levels. Hyper-systolic sounds were encountered at first, but were not present after the back of the stethoscope head was covered with felt. They resulted from the transmission of the sounds produced by the impact of the column of blood on the upper edge of the cuff through the compressed air of the inflated cuff.

*Comments.*—Since dogs exhibit marked arrhythmia, the blood pressure may vary with each respiration over a range of several millimeters of mercury. The accuracy of the method has been ascertained by taking with the auscultatory method readings in one hind limb simultaneously with readings taken with the Hamilton manometer in the other hind limb. Discrepancies of more than 10 mm. Hg in the systolic and diastolic levels between the two methods were uncommon.

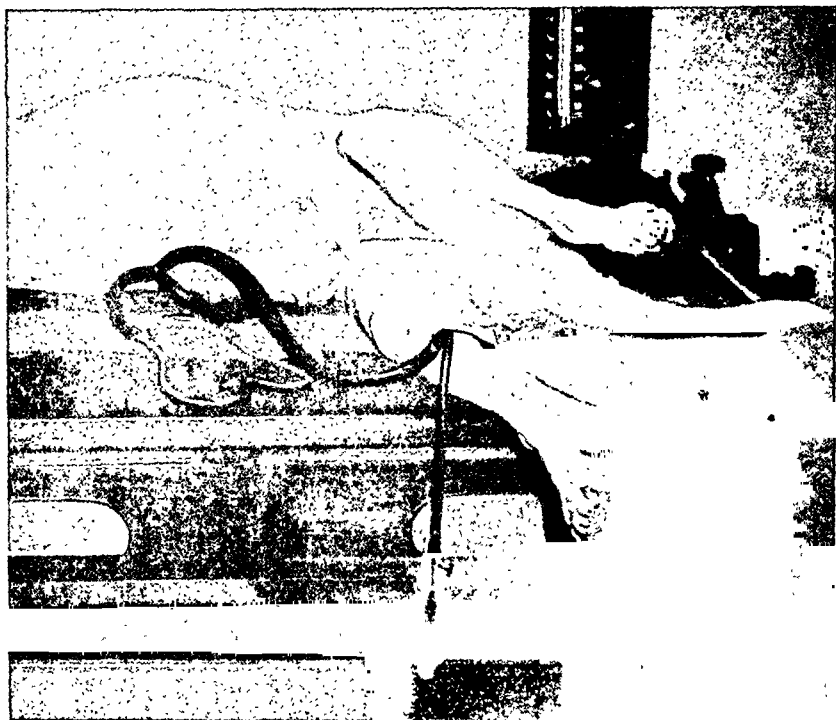
The blood pressure in nine animals has been followed over a period of several months. A routine was established: the animals were allowed the freedom of an outdoor pen for a few minutes, were then placed on a table in a quiet room, and were rested a few minutes before readings were recorded. The levels fell during the first two weeks, as the animals became accustomed to the procedure. afterwards, they appeared to enjoy the routine and would remain quiet. Variations in the routine, the presence of strangers, and the occurrence of strange

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noises were accompanied by higher readings. The blood pressure levels of these dogs approximates the levels reported by other investigators, for example, 138 mm. Hg systolic and 79 mm. Hg diastolic.<sup>1</sup>



*Conclusions.*—1. A modification of the auscultatory technique for the estimation of the blood pressure of dogs is described.

2. The value of a routine in following the blood pressures is emphasized.

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## CHEMICAL

### A METHOD FOR SIMULTANEOUS DETERMINATION OF METHEMOGLOBIN-OXYHEMOGLOBIN RATIO AND PER CENT HEMOLYSIS IN STORED BLOOD\*

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THE concentration of plasma hemoglobin may be used in determining the amount of hemolysis in blood. The extinction (negative logarithm<sub>10</sub> of transmission) at  $\lambda$  540 m $\mu$  can be taken to calculate directly the oxyhemoglobin concentration, in terms of a similar measurement on totally hemolyzed whole blood of known hemoglobin content. With a spectrophotometer such a method can be very precise, provided the hemoglobin has not undergone any change, such as oxidation. Alternative procedures involve conversion of the hemoglobin to acid or alkaline hematin, methemoglobin, or other derivatives (Peters and Van Slyke, 1932), with spectrophotometric measurement at appropriate wave lengths.

In the course of experiments on stored human blood in this laboratory, it became necessary to make routine, multiple measurements on small samples and to determine simultaneously both hemolysis and the relative amount of plasma hemoglobin present as methemoglobin. The latter pigment was formed in rather high concentration under certain experimental conditions and the methemoglobin-oxyhemoglobin ratio therefore assumed significance in our work. The clinical method described by Wendel (1938), although simple and sufficiently accurate, measures only methemoglobin, unless additional procedures are employed. Ray, Blair, and Thomas (1932) give details for qualitative and quantitative determination of blood pigments, either singly or in mixtures, by spectrophotometry; and Austin and Drabkin (1935) report a detailed study of the absorption spectrum for various known ratios of methemoglobin to oxyhemoglobin, measured at different pH's.

The procedure which was devised for the present work permits estimation of the ratio of methemoglobin to oxyhemoglobin in the sample by a simple graphical comparison method. The amount of oxyhemoglobin that would have been present if none had been oxidized to methemoglobin can subsequently be calculated, if desired for a determination of hemolysis.

*Methods.*—Spectrophotometric measurements were made with a Coleman spectrophotometer, Model 10-S (Coleman Electric Co., Maywood, Ill.), and the procedure was as follows:

About 0.7 c.c. of whole blood was used for a single determination. The cells were separated from the plasma in an air turbine, handling tubes of

\*From the Physiological Laboratory, Princeton University.

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about 0.8 c.c. volume and developing a force of about 20,000 G (gravity). Ordinarily 0.10 c.c. of the supernatant was diluted with 12.5 c.c. of a salt-buffer mixture\* before measurement.

As will be shown, methemoglobin is the only derivative of hemoglobin which appeared in the blood under the conditions of our work. Consequently, the spectrophotometric measurements were designed to pick up either oxyhemoglobin or methemoglobin. Any reduced hemoglobin present in the original blood sample was always automatically oxygenated during the dilution of the plasma.

The pII of the plasma, as diluted 1:125 with the salt-buffer mixture, is 7.3. Consequently, any methemoglobin present exists as neutral methemoglobin, and no error arises due to changes in the absorption spectrum of methemoglobin with changes in pH. (Austin and Drabkin, 1935.)

We routinely measured the ratio of methemoglobin to oxyhemoglobin in the following way. The negative logarithm of the transmission of the diluted plasma was determined at seven wave lengths ( $\lambda = 631, 600, 577, 560, 540, 520$ , and  $500 \text{ m}\mu$ ), which include the principal absorption bands of oxyhemoglobin and methemoglobin. The logarithms of these values were then plotted as the ordinate on transparent graph paper, against the wave length as the abscissa. By superimposing on calibration curves representing known ratios of methemoglobin to oxyhemoglobin, plotted similarly, it was possible to ascertain by inspection the percentage of hemoglobin that had been converted to methemoglobin in the experimental sample. Use of the log plot automatically cancels out any absolute concentration differences between solutions.

The calibration curves were made as follows. Fresh whole blood was accurately diluted 1:250 with salt-buffer mixture, causing complete hemolysis. To 100 c.c. of the resulting solution a small crystal of potassium ferricyanide was added, which oxidized the hemoglobin to methemoglobin. Four samples of this solution were diluted with salt-buffer mixture in such a way that the resulting solutions contained, respectively, 80 per cent, 60 per cent, 40 per cent, and 20 per cent of the methemoglobin concentration of the original solution. The extinction (negative logarithm<sub>10</sub> transmission) of these four solutions and of the undiluted original solution was determined at the seven wave lengths specified above.

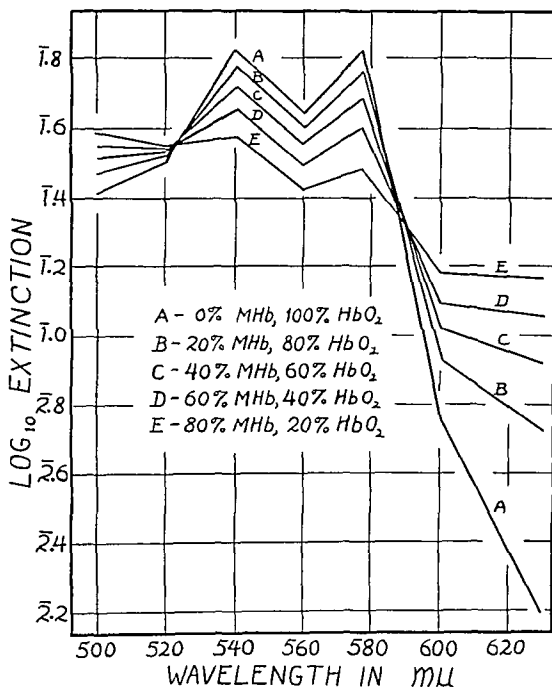
In the same way, samples of the original solution of hemolyzed whole blood (1:250) were diluted with salt-buffer solution lacking ferricyanide, and their extinctions were determined at the same seven wave lengths. It was now easy to construct arithmetically the curves of extinction against wave length for mixtures of oxyhemoglobin and methemoglobin in which these two components would be present in the following ratios: 100 parts oxyhemoglobin to 0 parts methemoglobin, 80 to 20, 60 to 40, 40 to 60, 20 to 80, and 0 to 100. The logarithms<sub>10</sub> of these calculated extinction values were next found and plotted against the wave length. By superimposing an experimental seven-point absorption spectrum, plotted as log extinction, on these standard absorption spectra of known oxyhemoglobin-methemoglobin mixtures, similarly

\*17.5 volumes of 0.06 M disodium phosphate, 7.5 volumes of 0.06 M monopotassium phosphate, 25 volumes of 0.06 M sodium chloride, and 50 volumes of water. The pH of this solution is 7.25. The transmission of the constituents of the plasma remains constant over many hours when diluted with such a solution (Parpart, Amberson, and Stewart, 1931).



plotted, the percentage of hemoglobin that was in the form of methemoglobin could be determined.

Our standard calibration curves are shown in Fig. 1, and the extinction values from which they were computed are given in Table I. The use of a seven-point spectrum, rather than one with fewer points, increases the precision, since the curves can be fitted fairly exactly, even though one or more points may be in error.



of calibration data representing the relative amounts in standard mixtures. The experimentally measured blood samples can be matched against this graph, by determine the percentage of the hemoglobin that is in the

If, in addition to the plasma methemoglobin-oxyhemoglobin ratio, the percentage of hemolysis is also required, this can be calculated. An example will suffice to illustrate the method. If the experimentally measured extinction of the diluted blood plasma sample at 540  $m\mu$  were 0.525, and 60 per cent of the hemoglobin were found from the calibration curves to be in the form of methemoglobin, then it would be necessary to multiply the observed extinction at 540  $m\mu$  by 1.46 to obtain the extinction that would have been

measured experimentally if all of the pigment had been present as oxyhemoglobin. The factor, 1.46, is obtained by dividing the extinction at 540  $m\mu$  of the 100 per cent hemoglobin calibration curve by the extinction at 540  $m\mu$  of the 60 per cent methemoglobin-40 per cent oxyhemoglobin calibration curve. These factors for various percentages of hemoglobin as methemoglobin are shown graphically in Fig. 2.

TABLE I

EXTINCTION VALUES FOR STANDARD DILUTIONS OF TWO SOLUTIONS, ONE CONTAINING OXYHEMOGLOBIN, AND THE OTHER CONTAINING METHEMOGLOBIN; THE CALIBRATION GRAPHS IN FIG. 1 WERE COMPUTED FROM THESE DATA, AS DESCRIBED IN THE TEXT

SOLUTION	WAVE LENGTH IN $m\mu$						
	500	520	540	560	577	600	631
Oxyhemoglobin "undiluted"*	.254	.317	.666	.441	.674	.059	.015
Above solution diluted 20 per cent	.208	.263	.530	.354	.542	.048	.014
Diluted 40 per cent	.156	.192	.399	.266	.406	.037	.011
Diluted 60 per cent	.106	.133	.272	.179	.275	.026	.008
Diluted 80 per cent	.054	.068	.136	.091	.137	.016	.006
Methemoglobin "undiluted"†	--	--	--	--	--	--	--
Above solution diluted 20 per cent	.331	.288	.246	.173	.168	.136	.142
Diluted 40 per cent	.249	.215	.184	.130	.127	.099	.107
Diluted 60 per cent	.168	.146	.126	.090	.088	.069	.073
Diluted 80 per cent	.089	.077	.068	.049	.046	.038	.040

\*0.050 c.c. of whole blood and 12.5 c.c. of salt-buffer solution.

†Same as above, but containing potassium ferrieyanide.

The red cells of stored blood frequently undergo very marked changes in volume. This varies the distribution of water between the cells and plasma. It is therefore necessary, in determining the degree of hemolysis in stored blood, to correct the hemoglobin measurements made on the plasma for these shifts of water. In addition, the degree of hemolysis itself affects the relative volume occupied by cells and plasma. Unless these factors are taken into account, the measurement of the percentage hemolysis can be greatly in error.

The method for making these corrections is as follows: The cell-free volume can be determined accurately by hematocrit measurements. These were made by a specially devised method which gives very precise and reproducible values with a standard deviation of 0.34 per cent (Parpart and Ballentine, 1943). Having determined from the hematocrit reading what percentage of the total volume of the experimental blood sample represents extracellular fluid (i.e., plasma), the calculated extinction value of the oxyhemoglobin found for the plasma is multiplied by the per cent of the total volume of the sample which is not occupied by cells. For example, if the hematocrit shows that 40 per cent of the total blood volume is occupied by cells, then the factor would be 60 per cent, or 0.60. The final corrected extinction is proportional to the concentration of oxyhemoglobin that would have been measured in the sample had the cells been replaced by an equal volume of water. Therefore, a direct comparison can be made between the extinction at 540  $m\mu$  of the diluted, hemolyzed original blood, before storage or treatment, and the extinction (at 540  $m\mu$ ) of the diluted plasma at any time. The latter figure (corrected for methemoglobin present, hematocrit value, and any differences in dilution) can be divided by the former to give a measure of percentage of hemolysis.

Before reliance can be placed upon the method as a whole, it is necessary to demonstrate two things. First, it must be shown that during the application of the high centrifugal force necessary to pack the cells for removal of the plasma sample, no change in the concentration of hemoglobin in the plasma occurs, because of possible mechanical destruction of cells, or evaporation of water from the plasma. Second, it must be shown that during the time the hemoglobin remains in the plasma following progressive cell breakdown during storage (in some experiments as long as six weeks at room temperature), the hemoglobin does not undergo change to anything other than methemoglobin, since the method of measurement is designed for quantitative detection of oxyhemoglobin and methemoglobin only.

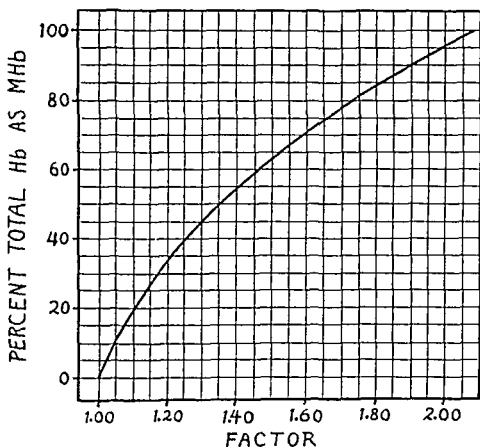


Fig. 2.—Factors by which the observed extinction values at  $\lambda$  540  $m\mu$  are multiplied to give the extinction values that would have been measured if all the hemoglobin had been in the form of oxyhemoglobin and none in the form of methemoglobin. These factors were calculated from the data of Table I as described in the text.

To test the first point, the transmission at  $\lambda$  540  $m\mu$  of diluted plasma from blood that showed about 30 per cent hemolysis (and whose cells were presumably less resistant to mechanical strain than when the blood was freshly drawn) was measured after various centrifugation times of from five to sixty minutes. No significant change in transmission was found for times of thirty minutes or less, although a slight decrease (about 1 per cent) in transmission occurred when the time was prolonged to sixty minutes. Since, in our experimental procedure, the centrifugation time is fifteen minutes, no error arises because of this factor.

The possibility that plasma hemoglobin might be transformed to derivatives other than oxyhemoglobin and methemoglobin during long periods of storage can also be tested. If all the hemoglobin originally present remains as oxyhemoglobin or methemoglobin and if none of it is transformed to other

derivatives during the time that it remains in the plasma following spontaneously occurring hemolysis of some of the cells, then there should be no change in extinction at  $540\text{ m}\mu$  (corrected only for methemoglobin present) when a sample of the blood is completely hemolyzed and measured at various times after storing. On the other hand, if loss of plasma hemoglobin occurs because of conversion to forms other than oxyhemoglobin or methemoglobin, then the extinction ( $540\text{ m}\mu$ ) of the blood (corrected for methemoglobin), measured after completely hemolyzing the cells, should be appreciably different than it was when the blood was stored. This change should be greater with greater degrees of hemolysis. Table II gives measurements on four samples of stored blood to test this point. Each sample was subjected to a different treatment. The table shows (1) the extinction of a totally hemolyzed sample of the fresh blood from which the samples were taken for storage; (2) the extinction (corrected for methemoglobin) of the totally hemolyzed blood samples after twenty-eight days of storage, and (3) the percentage hemolysis in each sample at the time the second measurement was made. Dilution factors are the same in all cases. It is apparent that there has been no significant change in extinction (corrected for any methemoglobin present) during the storage period. Identical results have also been obtained in numerous other samples. It is safe to conclude, therefore, that no derivatives of hemoglobin other than oxyhemoglobin and methemoglobin appear in the plasma or cells during storage or as the result of the various experimental treatments to which the blood has been subjected in our procedures.

TABLE II

DEMONSTRATION THAT THERE IS NO SIGNIFICANT LOSS OF HEMOGLOBIN BY CONVERSION TO DERIVATIVES OTHER THAN METHEMOGLOBIN, IN PLASMA FOLLOWING SPONTANEOUS, PARTIAL HEMOLYSIS OCCURRING DURING 28 DAYS OF BLOOD STORAGE

SAMPLE NO.	PER CENT HEMOLYSIS	MEASURED EXTINCTION OF TOTAL HEMOGLOBIN ORIGINALLY PRESENT	CALCULATED EXTINCTION OF TOTAL HEMOGLOBIN AFTER 28 DAYS' STORAGE
132	34	0.666	0.672
134	33	0.666	0.667
136	31	0.666	0.668
139	8	0.666	0.681

Because of the similarity of the absorption spectrum of methemalbumin (Fairley, 1939, 1941) to that of methemoglobin, it seemed advisable to test specifically for methemalbumin in plasma of stored blood samples selected at random. Treatment with 5 per cent sodium cyanide and subsequent spectrophotometric examination (Fox, 1941) failed to show the presence of this abnormal blood pigment.

*Discussion.*—It can be concluded that the measurement of hemoglobin as oxyhemoglobin and methemoglobin can be relied on to give data that permit a valid determination, with a single small blood sample, of per cent hemolysis and of the methemoglobin-oxyhemoglobin ratio. The precision of the method for determination of the plasma methemoglobin-oxyhemoglobin ratio depends essentially upon the concentration of the cell pigments in the plasma sample. When this is such that a 1:125 dilution of the plasma gives a solution whose transmission at  $\lambda = 540\text{ m}\mu$  is between 0.8 and 0.2, a difference of 10 per cent

in the percentage of total hemoglobin present as methemoglobin can be readily detected. This represents the upper limit of precision of the method under ordinary conditions. It is essential, of course, that no abnormal colored substances be present in the blood. If the hemolysis is less than about 3 per cent, the plasma methemoglobin-oxyhemoglobin ratio can be determined accurately only if the plasma is diluted considerably less than 1:125. In this case a correction must be made for the color of the normal plasma components. Where only a very small amount of blood is available for measurement, it is impossible to use dilutions much less than 1:125, unless special absorption cells of small volume but undiminished optical depth are available. The Coleman spectrophotometer is not easily adapted to the use of such cells. However, in determining percentage hemolysis from spectrophotometric measurements of plasma oxyhemoglobin, it is unnecessary to make any correction for hemoglobin present as methemoglobin when the hemolysis is less than 3 per cent, since only a very small error will result from omitting the correction under such conditions. The correction becomes increasingly important as the percentage of hemolysis increases beyond this value.

Measurement of the methemoglobin-oxyhemoglobin ratio in totally hemolyzed whole blood (plasma plus cells) can always be made with the maximum precision of the method, since the pigment concentration is here not a limiting factor. However, it has been our experience that in stored blood significant methemoglobin formation occurs only in the plasma.

#### SUMMARY

A method is described for measuring, simultaneously, on a small sample of stored blood the percentage of the hemoglobin in the plasma or whole blood that is in the form of methemoglobin, and the percentage of hemolysis. Because of the small volume of blood required, routine, multiple determinations can be made.

It is shown that the method does not suffer from errors due to possible injury of the cells during the centrifugation required to obtain the plasma sample, or due to possible conversion of the hemoglobin to derivatives other than oxyhemoglobin and methemoglobin during the period of storage.

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# A NEW TECHNIQUE FOR THE MEASUREMENT OF GLYCOGENESIS: A STUDY OF GLYCOGEN METABOLISM IN THE LIVER UNDER FASTING CONDITIONS\*

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THE conventional method for the study of glycogenesis involves two steps, namely, the determination of the liver glycogen of a control group of animals fasted for a certain time; and the estimation of the liver glycogen of another group of animals following the administration of a test substance. The difference between the mean of the control and the test group is regarded as the effect produced by the intermediary procedure. In such a method it is obvious that a large number of animals must be used in order to compensate for the marked individual variations observed in liver glycogen. It is also evident that a large group of animals would require that a considerable quantity of the material be tested.

In an investigation of the metabolism of a rare sugar (mannoheptulose) in this laboratory it was desired to study glycogen formation. Since there is evidence that this sugar is utilized by the rabbit and not by the rat as indicated by blood studies of Roe and Hudson,<sup>1</sup> it was necessary that a method for the determination of glycogenesis in the rabbit be developed in which small quantities of test substance would suffice.

A technique has been developed in which each animal serves as its own control. A laparotomy is performed under nembutal anesthesia, a control sample of liver is taken, the operated area is closed, and the test substance is injected subcutaneously. After waiting an appropriate time, a second laparotomy is made under nembutal anesthesia and samples of liver are taken for analysis. Thus, a significant result is obtained upon a single animal, and a satisfactory answer to the problem is offered by the use of relatively few animals and a small amount of test substance.

The effective use of the new technique requires that information should be available on the control values of glycogen in normal fasted animals and upon the effect of the operative procedure on normal animals. This double laparotomy technique might involve a change in liver glycogen due to (a) the effect of anesthetic and (b) the effect of surgery. The utilization of this technique in the determination of the effect of administered substances upon the production of glycogen allows the investigator to rule out the combined effect of anesthetic and surgery. Guest<sup>2</sup> has used nembutal as an anesthetic with rats. He states ". . . the high glycogen concentrations, 'normal' blood sugar and lactic acid concentrations are indicative that glycogenolysis with this anesthetic has been minimal." There is, however, a remote possibility that the use of nembutal

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does allow a change in liver glycogen. The work of Guest would not preclude the possibility that glucose could be utilized by metabolic processes at the same rate that it would be furnished from glycogen stored in the liver and would, over a period, tend to reduce the amount of glycogen stored.

We have determined the amount of glycogen present in the liver after an indicated fasting period and have also found the amount of glycogen present in the same liver after an additional twelve-hour interval. The difference between these values includes both the effect of surgery and of anesthetic. If nembutal is the anesthetic used and the surgical technique is similar each time, the normal amount of glycogen formed during the test interval is known and may be used as a base line value in the estimation of glycogenesis. This amount of glycogenesis is compared with the amount which results from the administration of other substances.

*Operative Procedure.*—Previous to the fasting period, the rabbits were maintained on a standard uniform diet consisting of rabbit chow.\* The rabbit was fasted a designated period (see Table I). At the end of this fasting period, the animal was given nembutal intraperitoneally (30 mg. per kg. of body weight). If necessary, additional small amounts of nembutal were injected into the marginal ear vein in order that the animal might become completely relaxed. A small incision was made along the midline of the abdomen. A small section (0.8 to 1.5 Gm.) of the edge of the right lobe of the liver was cut off with a scissors and placed immediately in a tared tube containing cold 30 per cent KOH. Skin clips were quickly clamped into the incised region of the lobe to aid in the control of bleeding. Very little bleeding occurred. The animal was closed and placed in a metabolism cage. The effects of the anesthetic wore off within three to four hours. The animal sat up, moved about in the cage, and took water. Throughout the remainder of the twelve-hour period the rabbit was continued in the fasting condition and was given water ad libitum. The sample of liver obtained by this first operation constituted the "control" sample on which liver glycogen was determined.

Twelve hours after the first operation (described above), the animal was again anesthetized with a full dose of nembutal (30 mg. per kg. of body weight). Care was taken not to excite the animal during this procedure. The abdomen was again opened and a small section of liver was cut from the intact left lobe. A second sample of liver was removed from the previously operated right lobe, not immediately in the operated area. A comparison could thus be made of the glycogen content of the operated and nonoperated lobes. The samples of liver were placed in tared tubes containing 30 per cent KOH and were analyzed for their glycogen content. The glycogen was estimated by the method of Good, Kramer, and Somogyi.<sup>3</sup> The glucose formed on hydrolysis of the glycogen was determined by the method of Shaffer and Hartmann.<sup>4</sup> The glycogen values, expressed as per cent glucose, are given in Table I.

*Data Obtained as a Result of Operative Procedure.*—In Table I are listed in the first column the tabulation number given to the experimental animal, in column 2 the weight of the experimental animal in kilograms, in column 3 the time in hours for which the rabbit was fasted prior to the first operation; column 4 under the heading "Control" Rt. Lobe indicates the values of glycogen

\*The chow was purchased from the Maritime Milling Company, Buffalo, New York.

expressed as per cent of liver weight. (The right lobe was selected as the lobe on which the initial operation was performed.) In column 5 is given the mean value of glycogen for all of the experimental animals which were fasted the indicated time prior to the initial operation. In column 6 is given the time interval between the beginning of the fast and the second operation. In column 7 are presented the liver glycogen values for the left lobe at the time of the second operation. Corresponding values for the right lobe are given in column 8. Column 9 contains the mean values for the glycogen content in per cent at the time of the second operation.

TABLE I  
LIVER GLYCOGEN OF FASTED RABBITS

COL. 1	2	3	4	5	6	7	8	9
RABBIT NO.	WT. IN KG.	HRS. FASTED PRIOR TO FIRST OPERATION	GLYCOGEN PER CENT (EXPRESSED AS GLUCOSE)					MEAN GLYCOGEN AFTER SECOND OPERATION
			"CON-TROL" RT. LOBE	MEAN OF "CON-TROL" RT. LOBE	HRS. FASTED PRIOR TO SECOND OPERATION	12 HR. AFTER FIRST OPERATION		
						LEFT LOBE	RIGHT LOBE	
26	1.98	12	3.01		24	1.33	0.943	
28	1.80	12	5.40	4.2	24	1.44	1.52	1.31
31	2.05	18	0.30		30	0.756	0.692	
32	1.81	18	0.559		30	0.85	0.795	
37	2.15	18	0.331		30	0.30	0.515	
38	2.00	18	0.207	0.35	30	0.451	0.464	0.60
21	1.98	24	0.224		36	0.86	0.91	
22	2.03	24	0.325		36	0.68	0.96	
23	1.83	24	0.40		36	1.52	2.50	
25	2.22	24	0.28		36	1.45	0.99	
39	1.88	24	0.537		36	-----	-----	
36	1.75	24	0.50		36	-----	-----	
40	1.94	24	0.437		36	0.388	0.525	
41	1.90	24	1.09		36	-----	-----	
42	1.98	24	0.839	0.52	36	-----	-----	1.08
29	2.01	36	0.188		48	1.93	2.35	
30	1.71	36	0.349		48	1.33	1.35	
34	2.00	36	0.10		48	2.20	2.04	
35	1.80	36	0.20		48	1.57	1.63	
43	2.30	36	0.449		48	1.45	1.16	
44	2.63	36	2.20		48	1.51	1.17	
46	2.33	36	0.407	0.56	48	0.70	0.62	1.50
8	1.95	48	1.13		60	-----	-----	
9	2.20	48	0.507		60	-----	-----	
10	1.89	48	0.983		60	-----	-----	
11	3.29	48	0.554		60	-----	-----	
33	2.31	48	1.66		60	3.89	3.56	
47	1.71	48	0.404		60	0.777	0.646	
48	2.51	48	0.721		60	0.265	0.172	
49	2.35	48	0.53		60	0.497	0.416	
50	2.61	48	1.58	0.90	60	0.782	1.025	1.20
51	2.74	60	0.57		72	1.53	1.51	
52	2.44	60	0.267		72	0.965	0.992	
53	2.49	60	0.905		72	2.265	2.142	
54	2.68	60	1.150		72	1.82	1.88	
55	2.77	60	1.090	0.80	72	1.82	1.42	1.63

*Glycogen Content of Liver Lobes Twelve Hours After the First Operation.*

—It will be noted from the values presented in columns 7 and 8 of Table I that the glycogen values of the right lobe are approximately equal to those of the left lobe; in fact, in many cases exceed those of the left lobe. Since the operation



was performed on the right lobe, an equal or higher glycogen content in this lobe is an indication that the operative technique has not impaired the glycogenetic function of either the operated lobe or of the liver as a whole.

The data represented in Table I are shown graphically in Fig. 1. Curve A is the curve which represents the amount of glycogen at the time of the initial operation. The abscissa indicates the number of hours which have elapsed between the beginning of the fast and the initial operation. Curve B represents the values for liver glycogen which were obtained the indicated number of hours after the beginning of the fast, but this time interval includes a 12-hour period after the initial operation. It is evident that the vertical distance between the curves A and B for any given time represents the change in liver glycogen to be attributed to the surgical procedure including the effect of the anesthetic. The circles represent the average values obtained at the indicated times for the initial operation, whereas the crosses represent similar values for the second operation.

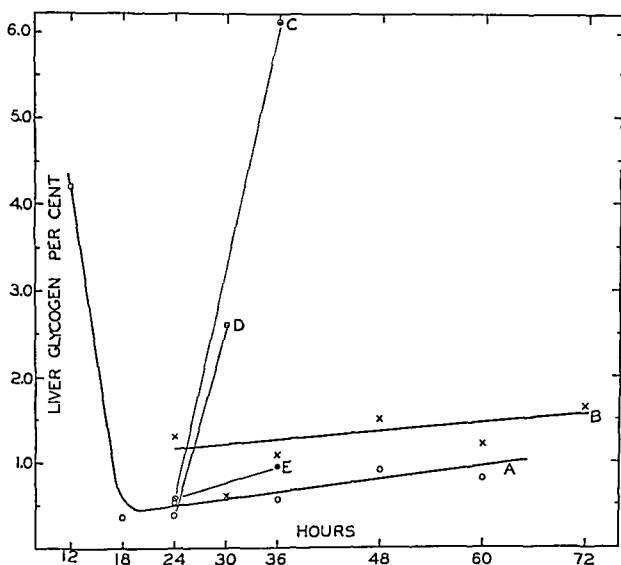


Fig. 1.—Liver glycogen values obtained after indicated period (12 hours after the initial operation; glucose was administered immediately after the initial operation). Curve D, liver glycogen values obtained 6 hours after the initial operation (12 hours before the final operation).

Curve B, average of liver glycogen values after the indicated period (12 hours after the initial operation; glucose was administered immediately after the initial operation; 6 hours after the second operation; 6 hours after the final operation). Curve C, liver glycogen values obtained 6 hours after the initial operation (12 hours before the final operation).

**Liver Glycogen Changes During Fasting.**—Curve A of Fig. 1 represents the change of liver glycogen in terms of per cent by weight of liver. Since the points indicated are average values, it shows that during the first 18 hours after

the beginning of the fast the glycogen content diminishes quite rapidly. After this interval, the effects of ingested food become submerged, and there appears a slow but definite increase in the glycogen content of the liver of the rabbit.

From the data presented in Fig. 1 it would appear that the most favorable time to measure glycogenesis in the rabbit is from 24 to 36 hours after withdrawal of food. By this time the effects of glycogen storage from previously ingested food are no longer evident and the slight increase in glycogen presumably from protein breakdown has become established. A longer fasting period gives no advantage from the standpoint of obtaining a more stable glycogen level, but does have the disadvantage of a more weakened experimental animal.

*Rate of Increase of Glycogen.*—During the 12-hour interval after the initial operation there is a change in the amount of glycogen present in the liver. The changes in the normal animal are due to normal metabolic processes combined with the effect of the operation. Should a substance under investigation be administered, it would be expected that the amount of glycogen formed during a 12-hour interval would be different. Since there is a difference between the amount of glycogen present at the beginning of the 12-hour interval and the amount at the end, the changes observed can be viewed as a rate of change per unit time, for example, the grams of glycogen per 100 grams of liver per hour. In Table II are indicated the rates of increase of liver glycogen for various intervals of fasting preceding the initial operation. These rates have been calculated by using the average values of glycogen at initial operation and the average values of the second operation (12-hour interval between operations). It is interesting to observe that there is an initial decrease in glycogen as indicated by the negative value of the rate of change, and that subsequently there appears to be a rate of increase which is roughly constant within experimental error.

TABLE II

AVERAGE RATE OF INCREASE OF GLYCOGEN DURING THE 12-HOUR PERIOD AFTER THE INITIAL OPERATION FOR VARYING PERIODS OF FASTING

HR. OF FAST PRECEDING INITIAL OPERATION	RATE OF INCREASE IN GM. OF GLYCOGEN PER 100 GM. LIVER PER HR.
12	-0.241
18	+0.021
24	+0.046
36	+0.079
48	+0.025
60	+0.070

It can be seen from the curves in Fig. 1 that the glycogen values obtained 12 hours after the first operation for the fasting period between 24 and 36 hours closely parallel the values obtained on the control liver samples, but the values are all higher.

*Effect of Administration of Glucose and Mannoheptulose on Glycogenesis.*—To illustrate the operative technique and its use in indicating the effect of a glycogen-producing substance, the following experiments are reported. A control sample of liver was removed after a 24-hour fast. After the abdomen of the animal had been closed, an isotonic solution of dextrose (containing 3 Gm. per kg. of body weight) warmed to 37° C. was administered subcutaneously.

Twelve hours later samples from the right and left lobe of the liver were obtained. The values obtained in this experiment are indicated in Table III. These data are presented in graphical form in curve *C* of Fig. 1.

TABLE III

EFFECT OF GLUCOSE AND MANNOHEPTULOSE ON THE FORMATION OF LIVER GLYCOGEN IN THE RABBIT

SUBSTANCE INJECTED	AMOUNT INJECTED GM./KG.	RAB- BIT NO.	WT. IN KG.	HR. OF FAST	GLYCOGEN PER CENT (EXPRESSED AS GLUCOSE)				RATE OF INCR. GM. GLYCOGEN/ 100 GM./HR.	
					CONTROL RT. LOBE	HR. AFTER FIRST OPERATION				
						6		12		
						RT. LOBE	LEFT LOBE	RT. LOBE		LEFT LOBE
Glucose	3	12	2.0	24	0.23	2.65	2.80	-----	-----	+0.42
Glucose	3	41	1.9	24	1.09	-----	-----	7.90	4.08	+0.42
Mannohep- tulose	3	15	2.5	24	0.54	-----	-----	0.90	0.96	0
Average Control (9 animals)				24	0.52			0.98	1.16	+0.046

An additional experiment was performed in which the same procedure was used except that a 6-hour interval was allowed to elapse between the initial and final operations. The data are shown in Table III. These data are also shown graphically in Fig. 1, curve *D*. It will be noted that despite the difference in time, the rate of increase of liver glycogen was roughly the same, and this rate was higher than the rate of increase in the control rabbit by a factor of 10. That is, the rate of increase on the control animals fasted 24 hours measured by the operative technique described was 0.046 Gm. glycogen per 100 Gm. of liver per hour, whereas the rate of increase after glucose administration was 0.42 Gm. of glycogen per 100 Gm. of liver per hour.

The technique used for glucose administration was also followed when the rare sugar, d-mannoheptulose,\* was administered. The data are shown in Table III. From Fig. 1, curve *E*, it can be seen that mannoheptulose does not appear to be a glycogen-former in the usual sense, since the curve for mannoheptulose lies between curves *A* and *B* (the curves obtained from the two operations described). However, these data do not preclude the possibility that mannoheptulose may be a precursor of glycogen. Previous work<sup>1</sup> has shown that the administration of mannoheptulose to rabbits is followed by an increase in the yeast-fermentable copper-reducing substance of the blood. It is possible that the substance arising from the metabolic transformation of mannoheptulose is glycogenetic and yet is not formed fast enough to yield significant increases in liver glycogen.

## SUMMARY

1. A new technique for measuring glycogenesis has been developed. The method consists of a double laparotomy under nembutal anesthesia. At the first operation a sample of liver is obtained from a fasted animal to serve as a control; at the second operation a sample of liver is obtained which shows the effect of the test substance upon glycogenesis.

2. Surgery with nembutal anesthesia causes a mild increase in liver glycogen

\*Kindly furnished to us by Dr. C. S. Hudson of the National Institute of Health.

in rabbits fasted 24 to 60 hours. This increase has been determined and base line values have been established for the conditions of this method.

3. The method herein described applied to glucose and mannoheptulose gave highly significant results.

4. A study of the changing content of liver glycogen in rabbits under fasting conditions has been made.

5. In the rabbit, marked glycogenolysis occurs during the 12- to 18-hour period of fasting; during the 24- to 36-hour period after food withdrawal the glycogen content of the liver is fairly constant; at the 48- and 60-hour periods of fast the liver glycogen is measurably higher than during the preceding 24 hours.

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## MICRODETERMINATION OF BLOOD GLUCOSE\*

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A REVIEW of published methods for blood sugar determination failed to disclose one which fulfilled our requirements for a procedure simple enough so that a number of determinations could be carried out promptly and yet sufficiently precise for investigations where relatively small differences in blood glucose values were anticipated and where samples of only 0.1 c.c. of blood might be available for analysis. Existing methods, therefore, were modified and adapted to our needs.

The procedure evolved is a combination of parts of well-known and established methods. Protein removal is secured by the technique of Somogyi,<sup>1</sup> using an alkaline zinc solution. The nature of the reducing substance in the filtrate was shown to agree closely with the fermentable sugar or "true glucose" and was estimated by the photoelectric colorimeter after development of the Folin-Wu blue color.<sup>2</sup> The method is equally well suited to the visual colorimeter.

### REAGENTS

1. The alkaline copper tartrate and phosphomolybdic acid reagents for the Folin-Wu method are described in all standard textbooks on clinical chemistry.

2. Glucose solutions for standardization are made from a stock one per cent solution in a saturated aqueous solution of benzoic acid.

3. The zinc sulfate reagent is prepared by dissolving 6.25 Gm. zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) in about 200 c.c. water and then transferring to a liter volumetric flask. Sulfuric acid (62.5 c.c. of 0.25N solution) is added to the flask and mixed. The solution is made up to volume with water. On titration, 25 c.c. of this zinc sulfate solution should be neutralized by 5.00 to 5.10 c.c. of the 0.25 N sodium hydroxide solution (solution 4) using phenolphthalein as the indicator. Freshly prepared quantities of both of these solutions (3 and 4) should always be standardized in this way before being used.

4. Sodium hydroxide, 0.25 normal.

### PROCEDURE

For determinations on capillary blood, the finger is pricked with a sterile Hagedorn needle and held over a shallow paraffin cup so that the specimen can drip or be "milked" into this container. This paraffin cup is prepared according to Abrahamson<sup>3</sup> by pouring liquid paraffin into a medium-sized plastic or metal

\*From the Research Division of the New York Diabetes Association, Inc., and the Laboratory of Pathological Chemistry, Department of Medicine, New York Post-Graduate Medical School of Columbia University.

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bottle cap and cooling it to solidity rapidly, preferably in a refrigerator; on cooling and contraction, the surface becomes concave, forming the shallow cup. This cup has two distinct advantages. First, it serves to prevent rapid clotting; and, second, it allows several drops of blood to be pooled for pipetting without inclusion of air bubbles.

For measuring the blood, a serologic pipette calibrated to contain 0.1 c.c. blood is used. A convenient pipette filler can be assembled by inserting a well-fitting nut and bolt into a 3-inch length of heavy-walled rubber tubing. The opening of the tubing must fit tightly over the end of the pipette. When the tip of the pipette is dipped into the specimen, if airtight connections have been made, unscrewing the bolt will draw blood into the pipette.<sup>3</sup> Before discharging the measured specimen, the tip of the pipette is wiped with a clean piece of gauze.

The capillary or oxalated venous blood (0.1 c.c.) is discharged into 1.6 c.c. of the zinc sulfate reagent contained in a small test tube (100 × 13 mm.). The pipette is rinsed several times with the contents of this tube. The tube is shaken so that the blood is completely laked. The sample may be left at this stage, corked, at room temperature for several hours without danger of loss of sugar such as occurs with oxalated blood.

When it is convenient to proceed with the determination, 0.3 c.c. of 0.25 N sodium hydroxide is added and the test tube is vigorously agitated to assure uniform coagulation. After a few minutes' standing, it is centrifuged, and 1 c.c. of the clear, colorless supernatant fluid is transferred to a graduated 10 c.c. test tube (approximately 150 × 13 mm.). A similar tube receives 1 c.c. of standard glucose solution (0.005 or 0.01 per cent according to the anticipated blood glucose value). To each tube is added 1 c.c. alkaline copper tartrate reagent; the tubes are gently but thoroughly shaken and heated in a boiling water bath for exactly eight minutes in an upright position. The tubes are cooled in running water for one to two minutes, after which 1 c.c. of phosphomolybdic acid is added. All of the cuprous oxide should react with this reagent. The tubes must not be shaken at this point. If a scum of oxide persists on the surface of the blue solution, an additional drop of the phosphomolybdic acid solution should be allowed to fall on the film and cause its disappearance. The tubes should stand undisturbed for fifteen minutes for maximum color development.

The solutions are diluted to 6.25 c.c. or some other convenient volume, and the tubes are inverted several times. After four to five minutes, the colors are matched in a colorimeter. We have employed a Klett-Summerson photoelectric instrument fitted with a 42 filter (blue). If a visual colorimeter is used, micro cups and plungers should replace the standard equipment.

Special test tubes can be used for development of color. We have employed both constricted and plain tubes one-fourth the size of the regulation Folin-Wu sugar tube. The plain tube is superior to and less expensive than the constricted one. Stock 10 c.c. graduated tubes have been entirely satisfactory, however, and permit a wide range of dilutions.

## CALCULATION

For the Klett-Summerson photoelectric colorimeter:

$$\frac{\text{Reading of Unknown}}{\text{Reading of Standard}} \times 0.05 \times 2 \times 1000 = \text{mg./100 c.c.}$$

Where 0.05 = mg. glucose in 1 c.c. standard 0.005 per cent solution

2 = factor for aliquot of filtrate analyzed

1000 = factor for conversion of 0.1 c.c. to 100 c.c. blood

For the visual colorimeter: (standard set at 15 mm.)

$$\frac{15}{\text{Reading of Unknown}} \times 0.05 \times 2 \times 1000 = \text{mg./100 c.c.}$$

These equations are valid when dilution of standard and unknown are the same. If the dilutions differ, an additional factor must be used to correct the preceding equations.

If the photoelectric colorimeter is used, the results can be made more precise by running a blank, wherein 1 c.c. of water is substituted for the filtrate. The reading of this blank may be subtracted from all unknown and standard readings, or the pointer of the colorimeter may be set at zero with the blank and all other solutions read with the colorimeter at this setting. The use of the blank is impractical with the visual colorimeter, since the depth of color is too small for accurate reading.

## DISCUSSION

The method has been checked carefully for accuracy and reliability. Over two dozen specimens of capillary and venous blood have been analyzed in duplicate, and the results were found to check to within  $\pm 3$  mg. per 100 c.c.

In studying recovery with this method, 0.1 c.c., samples of pooled venous blood were analyzed in duplicate. Simultaneously, 0.1 c.c. samples of a 1:1 mixture of the same blood and 0.1 per cent glucose solution were tested. Values obtained agreed with the theoretical within the range of  $-1$  to  $+3$  mg. per 100 c.c.

Fermentation experiments were undertaken to ascertain how close the values obtained with this method came to "true glucose." The procedure of fermentation used was essentially that of Peters and Van Slyke,<sup>4</sup> except that a 0.5 c.c. portion of the yeast suspension was used for the 1 c.c. of filtrate. The findings are shown in Table I.

TABLE I  
COMPARISON OF "TRUE SUGAR" VALUES

SPECIMEN NO.	OBSERVED BLOOD SUGAR VALUE	FERMENTABLE SUGAR	DIFFERENCE
1.	71	69	+2
2.	75	74	+1
3.	343	340	+3
4.	264	262	+2
5.	98	97	+1

The yeast blank was found to give a reading equivalent to 1 mg. higher than the water blank. This correction has been made in the tabulated figures.

This micromethod was checked against the macromethod from which it was mainly devised. For several years the Laboratory of Pathological Chemistry

of the New York Post-Graduate Hospital has routinely used Somogyi's zinc filtrate (macro procedure II)<sup>1</sup> for the determination of blood sugar by the colorimetric technique of Folin and Wu.<sup>2</sup> The results of this combined macromethod were checked against the titration method which employs the Shaffer-Somogyi Reagent 50.<sup>5</sup> Colorimetric procedures have been found more satisfactory for routine conditions in a busy laboratory than titrimetric methods, especially those involving determination of blood sugar in finger-tip samples.

Comparison of the macromethod and the micro modification of this method were satisfactory. Deviations were both positive and negative, and none exceeded 5 mg. These comparative tests were not only run by a single analyst, but also by the routine laboratory against the special analyst.

#### SUMMARY

A micromethod for the accurate determination of true blood glucose is described. This method is suitable for routine and investigational purposes.

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## THE STANDARDIZATION AND ASSAY OF HEPARIN BY THE TOLUIDINE BLUE AND AZURE A REACTIONS\*

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### *A Correction*

**A**N error in the previous paper<sup>1</sup> has occurred with regard to the concentration of toluidine blue (TB) or azure A (AA), which concentration should be ten times more diluted. In Method I, it should read: Make up 1:10,000 toluidine blue or azure A by adding 1.6949 Gm. TB (NU-3) to 1,000 c.c. or 1.1494 Gm. AA (NAz-7) to 1,000 c.c. of distilled water, and further dilute these concentrations of TB or AA ten times in distilled water. In method II, it should read: A measured portion of the sample is titrated in a beaker with 1:20,000 solution of TB or AA made up by adding 0.8475 Gm. TB (NU-3) to 1,000 c.c. or 0.5747 Gm. AA (NAz-7) to 1,000 c.c. distilled water, and further dilute these concentrations of TB or AA ten times with distilled water.

Throughout the whole paper, all the concentrations of toluidine blue or azure A and all quantities given in gamma should read one-tenth of the reported values. Therefore, in the summary, the correction stands:

5. The colorimetric unit of heparin is defined. One unit of heparin de-colorizes 15 gamma of 100 per cent toluidine blue dye content. This unit equals 25.4 gamma of toluidine blue NU-3 or 17.2 gamma of azure A NAz-7.

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<sup>1</sup>Copley, A. L., and Whitney III, D. V.: The Standardization and Assay of Heparin by the Toluidine Blue and Azure A Reactions, *J. LAB. & CLIN. MED.* 28: 762, 1943.

Received for publication, Dec 8, 1943.

## BOOK NOTICES

### Texto de Bacteriología\*

ACCORDING to the authors, this book has been written for those who wish to understand the science of bacteriology: medical students, graduate students, technicians, etc. The material covered is divided into two parts: 36 of a total of 60 chapters are concerned with the historical background in detail, including biographical sketches of over 150 individuals, and the morphology and physiology of the bacteria; the remaining 24 chapters are devoted to apparatus and techniques. As an example of the thoroughness of this part, the electron microscope is considered in detail with reproductions of photographs of cocci, bacilli, and spirochetes taken with this instrument.

One misses in this text descriptions of the cultural and morphologic characteristics, biochemical and serologic reactions, and the pathogenicity of specific organisms.

English-speaking students wishing to acquire a vocabulary in Spanish of bacteriologic terms will find this book very helpful.

### Clinical Significance of the Blood in Tuberculosis†

ONE would wish to write only praise for a work into which enters so much toil and tribute by a trust fund. The paper book cover prints praises of authorities.

The book is vague and is not for technicians and is of questionable value for clinicians. The bibliography of some 604 references is far from complete. Important contributions on the subject made in recent years, which must be known to the author, are omitted. Poorly arranged, this book will prove confusing to the uninitiated in the field of blood work, since, instead of starting with a discussion of standards, thus laying a groundwork of understanding of what constitutes a normal blood, the author leaves this important subject until late in the book. These standards are arbitrarily set up without giving evidence as to rationale. Too few illustrations occur. Pictures of the blood cells under discussion are needed for practical purposes.

The chapter (27) on the correlation coefficient is extremely confusing and reveals a lack of understanding of higher statistical methods and the valuable assistance these methods can give in an investigation of hematological data in tuberculosis. It ignores a paper by Boissevain, Forster, and Good which suggests its foundation.

The changed leucocytic picture of altitude is neglected. In 1909, in *Colorado Medicine*, Webb and Williams first mentioned the lymphocyte increase in the blood due to altitude. This was later presented to and published by the Royal Society in England.

The increase in platelets in tuberculosis was well known to Osler, who always took the blood of a tuberculous patient to demonstrate platelets to his students. Research workers have done little to find out the why! Platelets are also increased by altitude.

Looseness of terms runs through the book. The best chapter deals with the sedimentation rate and the importance of securing a normal rate as well as a normal leucocytic response.

The section of the book devoted to the effect of therapeutic methods, such as pneumothorax and thoracoplasty and of exercise on the blood picture, may be valuable to a specialist treating tuberculosis.

GERALD B. WEBB.

\**Texto de Bacteriología*. By Arturo Curbelo y Hernandez, and Giraldo Insua y Cartaya. Professors Auxiliar y Agregado de la Catedra de Bacteriología de la Escuela de Medicina de la Universidad de la Habana. Cloth, illustrated with engravings and colored plates, 610 pages. M. V. Fresneda, Habana, Cuba, 1943.

†*Clinical Significance of the Blood in Tuberculosis*. By Gulli Lindh Muller, M.D., Pathologist and Formerly . . . New England Hospital for Women and Children, Boston; . . . State Sanatorium, Rutland, Massachusetts. Cloth, 516 pages. \$3.50. The . . . New York, 1943.

### Chronic Pulmonary Disease in South Wales Coalminers\*

THIS publication by the British Medical Research Council is entitled to the greatest praise. Forty-two cases of pneumoconiosis in coal miners are recorded with excellent x-ray photographs and pictured histologic sections.

Low relative humidity (deep mines) favored development of pneumoconiosis.

The lungs contained up to 6 Gm. of carbon and 2 Gm. of silica, mostly in the form of silicates, of which mica was the principal representative.

The term "dust reticulation" to correspond with x-ray reticulation is suggested to take the place of anthracosis. The latter term is unsuited, since the miners' lungs with reticulation have a notable retention of silica as well as carbon. A new word Koniophthisis is coined for a distinctive type of pulmonary tuberculosis modified by dust reticulation.

Incidence of pneumoconiosis is reported as four times as frequent in anthracite as in the bituminous miners, yet 14 per cent of the latter revealed x-ray shadows. Should such "dust reticulation" types be compensatable as are those of nodular types the industry will be faced with enormous claims.

These studies are well incorporated in a Report on Pneumoconiosis in The American Journal of Public Health, July, 1943, by the Committee investigating this disease.

GERALD B. WEBB.

### Micrurgical and Germ-Free Techniques†

THIS is an extremely interesting and well written book, but it is of little practical value to anyone outside of this narrow field. Descriptions, illustrations, and references are excellent. The information in chapters IX, X, and XI, on control of cross infection of airborne nature by various barriers, should be carefully weighed by anyone charged with design or remodeling of hospital wards and rooms, especially in relation to nursery planning.

Any student contemplating scientific research might read this book in order to develop a concept of what is meant by the term "scientific method."

### Borderlands of Psychiatry‡

THE "Borderlands of Psychiatry" include those diseases which are treated at times by the neurologist, again by the internist, and most frequently by the psychiatrist. Of these Dr. Cobb discusses stammering, epilepsy, the psychoneuroses, and the psychosomatic disorders. Cobb estimates that there are at least six million persons in this country who are troubled with one of these "borderland" disorders. This estimate if anything is a low one. The persons with psychoneurotic or psychosomatic disorders alone probably number higher than this, if one includes all persons with subclinical symptomatology. Indeed, one of our major medical and public health problems is to study and understand these disorders so that a start may be had in adequately treating and preventing them.

In *Borderlands of Psychiatry* Cobb presents an approach towards these frequently neglected diseases which should aid in dispelling much of the loose thinking which has centered about them and which has made them in general so poorly understood. This is a physiologic approach. He emphasizes that a priori acceptance of the validity of the freely used dichotomies, "organic and functional," "mental and physical," "psychic and somatic"

\*Chronic Pulmonary Disease in South Wales Coalminers I. Medical Studies. Privy Council. Medical Research Council. A Report by the Committee on Industrial Pulmonary Disease. B. Medical Survey, by P. D'Arcy Hart and E. A. Aslett, with contributions by D. Hicks and R. Yates. C. Pathological Report, by T. H. Belt with assistance from A. A. Ferris. Sixth Edition Paper, 222 pages, 10s. His Majesty's Stationery Office, London, 1942.

†Micrurgical and Germ-Free Techniques. Their Application to Experimental Biology and Medicine. A Symposium. Edited by James A. Reyniers, The Laboratories of Bacteriology, University of Notre Dame, Notre Dame, Indiana. Cloth, 274 pages, \$5.00. Charles C Thomas, Publisher, Springfield, Illinois and Baltimore, Maryland, 1943.

‡Borderlands of Psychiatry. By Stanley Cobb, M.D., Bullard Professor of Neuro-pathology, Harvard Medical School, and Psychiatrist-in-Chief, Massachusetts General Hospital, Cambridge, Mass. Harvard University Press, 1943.

is one of the chief factors leading to confusion in psychiatric and medical thought. He suggests that etiologic classification should be based on terms such as "psychogenic," "genogenic," "histogenic," and "chemogenic," and states that thereby it may be possible to avoid the intellectual pitfalls in which most of us are trapped when considering psychiatric disorders. He would have us deny the physiologically unsound tenets implied in the use of the terms "organic," "functional," etc.

This book, the fourth in a series of "Harvard University Monographs in Medicine and Public Health," is written in a conversational style and contains illustrative case histories and many thought-provoking personal views of Dr. Cobb. It can be recommended to physicians, medical students, and others for orientation in the complexities of psychiatry as it exists today.

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### Rabies\*

A VALUABLE arsenal of information concerning the status of rabies has been made available to the practicing physician, public health officer, and veterinarian. In the first portion, discussion is made of types of rabies, and the author proceeds in a full but rapid pace to the technique of laboratory examination. In the second part, all phases of control from state level down to the choice and evaluation of vaccine in human prevention are clearly presented. Of special interest is the treatment of the perplexing question of who shall take rabies treatment.

The bibliography and footnotes are very comprehensive. The appendix contains the meat of ordinances concerning rabies control for the health officer and, also, tables on immunizing potency of vaccines for the student.

Both the physician confronted with a case of dog bite and the health officer in the dilemma of a rabies epidemic will find rational *modi operandi* in this book.

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\*Rabies. By Leslie T. Webster, M.D., the Rockefeller Institute for Medical Research, New York. Cloth, \$1.75. The Macmillan Company, New York, 1942.

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## CLINICAL AND EXPERIMENTAL

### PERSISTENCE OF INFLUENZA VIRUS ON THE HUMAN HAND\*

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THE persistence of potent influenza virus in dried extraembryonic fluids of infected chick embryos reported in a preceding paper<sup>1</sup> of this series in conjunction with the earlier convincing report of Edward<sup>2</sup> regarding the persistence of influenza virus on soiled utensils at once suggested the possible agency of the human hand in the dissemination of this virus, particularly the hand which comes into contact with food and with utensils for serving food. The matter has already been studied by Krueger and his associates,<sup>3</sup> who observed rapid loss of potency in virus deposited on the hand and therefore concluded that transmission through this agency is not apt to be an important hazard.

Because contamination with the bacteria of the skin was to be expected in these experiments it was decided to use white mice rather than embryonated eggs in testing for active virus. The mice ranged in weight from 10 to 20 grams. Some were Swiss mice and others were unspecified white mice from dealers in laboratory animals. All seemed satisfactory for the tests. Inoculation was done by instilling several drops of virus suspension into the nostrils of the mouse under light ether anesthesia. A blunt 24 gauge needle attached to a tuberculin syringe was used.

*Experiment 1, January 20, 1943.*—Influenza virus of the Melbourne A strain which had been propagated in embryonated eggs was passed twice in series through mice. The lungs of a mouse of the second passage were ground and suspended in saline solution, allowed to settle, and the opalescent super-

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natant fluid was removed and inoculated directly, without drying, intranasally to four mice. All survived to be sacrificed on the ninth day. One mouse showed consolidation of three pulmonary lobes ( $9K_3$ ), one had two consolidated lobes ( $9K_2$ ), and two had one consolidated lobe ( $9K_1$ ). Some of the virus suspension was dried on glass by spreading 0.2 c.c. of it over the bottom of each of two sterile Petri dishes. After remaining dry for twenty minutes the material in one dish was taken up in 0.4 c.c. saline solution and inoculated intranasally to four mice. These were killed on the ninth day with positive findings; one mouse had small solid spots in the lungs ( $9K_+$ ), one had one lobe consolidated ( $9K_1$ ), another had two ( $9K_2$ ), and another had three consolidated lobes ( $9K_3$ ). The material dried in the other dish was kept for forty-eight hours and then resuspended in 0.4 c.c. saline solution and given to four mice intranasally. These were killed on the ninth day and revealed no evidence of infection ( $9K_0$ ,  $9K_0$ ,  $9K_0$ ,  $9K_0$ ).

*Experiment 2, January 27, 1943.*—A suspension of infected mouse lung in saline solution was used. Mrs. R. washed her hands with soap and water, rinsed them in running water, then with 95 per cent alcohol, and dried them under a sterile cloth for twenty minutes. Then the virus suspension, 0.2 c.c., was spread over an area of about 50 sq. cm. on the palm of the left hand, where it dried in twenty minutes. At the same time 0.2 c.c. of the same virus suspension was spread over a similar area in a Petri dish. After fifteen minutes in the dry state, each was resuspended in 0.4 c.c. saline solution and inoculated into mice. The four mice receiving material from the hand were killed on the twelfth day with the result being  $12K_3$ ,  $12K_1$ ,  $12K_2$ , and  $12K_+$ . The three mice receiving material from the dish were more seriously affected; one died on the fourth day with all lobes consolidated, another died on the twelfth day with extensive airless portions in all five lobes, and the third was killed on the twelfth day with all lobes involved ( $4D_5$ ,  $12D_5$ ,  $12K_5$ ).

*Experiment 3, February 18, 1943.*—Allantoic virus from eggs was used, with a titer of  $10^{-5}$ . Mrs. R. washed her hands with soap and water, rinsed in running water for five minutes, and then finally with 95 per cent alcohol. After the hands were entirely dry, the virus fluid, 0.2 c.c., was spread on the palm of the left hand where it became dry in fifteen minutes. After a further interval of ten minutes, the area was washed with 0.4 c.c. saline solution to resuspend the virus, and this material was inoculated into three mice. All died on the fourth day with consolidation in all five pulmonary lobes ( $4D_5$ ,  $4D_5$ ,  $4D_5$ ). At the same time the allantoic fluid in portions of 0.2 c.c. was spread in each of two Petri dishes and allowed to dry. One of these portions was resuspended after being dry for ten minutes, and the other after being dry for 200 minutes. The mouse results were  $4D_5$ ,  $4D_5$ ,  $5D_5$ , and  $4D_5$ ,  $4D_5$ ,  $4D_5$ , respectively. An end point was not reached and apparently not even remotely approached.

*Experiment 4, March 2, 1943.*—Allantoic virus was used. Miss S. washed her hands with soap and water, rinsed in running water for five minutes, and then quickly with 95 per cent alcohol. After drying by evaporation, each palm was spread with 0.2 c.c. of the virus suspension. After drying seemed to have been complete for ten minutes, the material on the left palm was taken up in 0.4 c.c. of saline solution, and after forty minutes that on the right palm was resuspended in saline. The control on glass was made in the usual way and

taken up in saline solution after remaining dry for forty minutes. The results in the mice were as follows:

Left palm, dry ten minutes	4D <sub>5</sub> , 4D <sub>5</sub> , 4D <sub>5</sub>
Right palm, dry forty minutes	3D <sub>5</sub> , 5D no autopsy, 8D <sub>5</sub>
Glass, dry forty minutes	3D <sub>3</sub> , 3D <sub>5</sub> , 5D <sub>5</sub>

Each entry shows, first, the day of death, second, D for natural death and K for killed, and, third, the number of pulmonary lobes involved. It is evident that the virus remained potent. As a control the normal allantoic fluid of embryonated eggs was introduced intranasally into three mice. These were killed on the tenth day and their lungs were normal.

*Experiment 5, March 11, 1943.*—Mrs. R. washed and dried her hands as she would habitually without any extra rinsing. Each palm was spread with 0.2 c.c. of allantoic virus, which dried after eight minutes. The same virus was also dried on glass. Mice inoculated in the usual way gave the following results:

Undried allantoic virus	4D <sub>5</sub> , 6D <sub>5</sub> , 18K <sub>2</sub>
Dried on glass forty minutes	4D <sub>5</sub> , 5D <sub>5</sub> , 6D <sub>5</sub>
Dried on hand ten minutes	4D <sub>7</sub> , 5D <sub>5</sub> , 18K <sub>0</sub>
Dried on hand forty minutes	5D <sub>5</sub> , 5D <sub>7</sub> , 5D <sub>5</sub>

One exceptional mouse appeared normal when killed on the eighteenth day (18K<sub>0</sub>). However, it is evident that potent virus persisted in this experiment.

*Experiment 6, March 17, 1943.*—Mrs. R. washed her hands in the habitual manner and rinsed them with very soapy water which was allowed to dry. Then allantoic virus, 0.2 c.c., was spread on each palm and allowed to dry under protection of a sterile cloth. The usual control on glass was made. Mice inoculated as usual gave the following results.

Dried on glass forty minutes	8D <sub>5</sub> , 8D <sub>5</sub> , 12K <sub>1</sub>
Dried on soapy hand ten minutes	12K <sub>0</sub> , 12K <sub>0</sub> , 12K <sub>0</sub>
Dried on soapy hand forty minutes	12K <sub>0</sub> , 12K <sub>0</sub> , 12K <sub>0</sub>

This result suggested that soap remaining on the skin might be a factor of significance in determining inactivation of the virus.

*Experiment 7, April 27, 1943.*—Mrs. R. washed her hands with soap and water, followed by rinse in running water for several minutes. Then one hand was given a final rinse with 95 per cent alcohol, and the other was finally rinsed in soapy water. The hands were then dried under a sterile towel for thirty minutes. Then allantoic virus of titer  $10^{-2}$  was spread, 0.2 c.c. on each palm and also as usual on glass. The mice gave the following results:

Undried virus undiluted	3D <sub>5</sub> , 5D <sub>5</sub> , 10D <sub>4</sub> , 11K <sub>3</sub>
Undried virus diluted 1:10	5D <sub>5</sub> , 11K <sub>1</sub> , 11K <sub>2</sub>
Dried on glass ten minutes, undiluted	5D <sub>5</sub> , 7D <sub>5</sub> , 8D <sub>5</sub>
Dried on glass ten minutes, diluted 1:10	5D <sub>5</sub> , 10D <sub>5</sub> , 10D <sub>5</sub>
Dried on alcohol palm ten minutes	11K <sub>0</sub> , 11K <sub>0</sub> , 11K <sub>0</sub>
Dried on soapy palm ten minutes	11K <sub>0</sub> , 11K <sub>0</sub> , 11K <sub>0</sub>

Evidently the virus remained potent on the glass but not on the skin. Possibly the low titer of the virus used ( $10^{-2}$ ) may have been a factor.

*Experiment 8, May 4, 1943.*—Mrs. R. washed her hands with soap and water and rinsed them in running water for several minutes. Then the left hand was given a final rinse with soapy water and the right hand was given a final rinse with 95 per cent alcohol. Both hands were held under a sterile cloth for thirty minutes, at the end of which time they were thoroughly dry. Allantoic virus, 0.2 c.c., was spread over about 50 sq. cm. on each palm and also on glass. The titer of the allantoic virus was found to be at least  $10^{-3}$  in eggs. Results in the mice were as follows:

Undried virus	3D <sub>5</sub> , 4D <sub>5</sub> , 6D <sub>5</sub>
Dried on glass thirty minutes	3D <sub>5</sub> , 4D <sub>5</sub> , 6D <sub>5</sub>
Dried on soapy hand ten minutes	10K <sub>0</sub> , 10K <sub>0</sub> , 10K <sub>0</sub>
Dried on alcohol hand thirty minutes	10K <sub>0</sub> , 10K <sub>5</sub> , 10K <sub>2</sub>

*Experiment 9, May 7, 1943.*—Mrs. R. washed her hands and rinsed them thoroughly. The left hand then given a final rinse in soapy water and the right hand a final rinse in 95 per cent alcohol. The hands were dried under a sterile towel for thirty minutes. Allantoic virus of titer  $10^{-5}$  was spread 0.2 c.c. on each palm and dried in seven minutes. After a further ten minutes the virus was taken up as usual in 0.4 c.c. saline solution. The usual control on glass was made. Results in the mice were as follows:

Undried virus undiluted	5D <sub>5</sub> , 5D <sub>5</sub> , 10K <sub>+</sub>
Undried virus diluted 1:10	4D <sub>5</sub> , 5D <sub>5</sub> , 6D <sub>5</sub>
Dried on glass ten minutes, undiluted	3D <sub>5</sub> , 4D <sub>5</sub> , 6D <sub>5</sub>
Dried on glass ten minutes, diluted 1:10	4D <sub>5</sub> , 5D <sub>5</sub> , 5D <sub>5</sub>
Dried on soapy hand ten minutes	10K <sub>0</sub> , 10K <sub>+</sub> , 10K <sub>0</sub>
Dried on alcohol hand ten minutes	5D <sub>5</sub> , 7D <sub>5</sub> , 7D <sub>5</sub>

*Experiment 10, May 17, 1943.*—Mrs. R. washed her hands as she would ordinarily and dried them on a paper towel. Each palm was then immediately spread with 0.2 c.c. allantoic virus. The left hand required seven minutes to dry and the right hand nine minutes. Controls on glass were made. Results in the mice were as follows:

Dried on glass thirteen minutes	5D <sub>5</sub> , 5D <sub>5</sub>
Dried on glass forty minutes	7D <sub>5</sub> , 10K <sub>0</sub>
Dried on right hand fourteen minutes	10K <sub>0</sub> , 10K <sub>0</sub> , 10K <sub>0</sub>
Dried on left hand forty-five minutes	10K <sub>+</sub> , 10K <sub>0</sub> , 10K <sub>0</sub>

*Experiment 11, May 20, 1943.*—Mrs. R. washed her hands as she would ordinarily and dried them on a paper towel. Then each palm was spread with allantoic virus 0.2 c.c. and both dried in ten minutes. The material was taken up from the right palm after a further ten minutes and from the left palm after forty-five minutes. The control on glass required forty-three minutes to dry and was taken up in 0.4 c.c. saline solution after a further 105 minutes. The original virus had a titer of  $10^{-2}$  in eggs. Results in the mice were as follows:

Undried virus diluted 1:10	4D <sub>5</sub> , 4D <sub>5</sub>
Dried on glass 105 minutes	4D <sub>5</sub> , 4D <sub>5</sub> , 4D <sub>5</sub>
Dried on right hand ten minutes	5D <sub>5</sub> , 8D <sub>5</sub> , 8D <sub>5</sub>
Dried on left hand forty-five minutes	5D <sub>5</sub> , 7D <sub>5</sub> , 9D <sub>5</sub>



In this experiment there was persistence of the virus on the skin, even though it was an allantoic virus of rather low titer.

*Experiment 12, May 29, 1943.*—Miss L. washed her hands as she ordinarily would and dried them with a paper towel. Then 0.2 c.c. of allantoic virus was spread on each palm. Drying required eight minutes. The left palm was sampled after seventeen minutes and the right after fifty minutes. The control on glass dried in sixty-five minutes and was sampled after a further thirty-one minutes. Titration of the virus in embryonated eggs failed because of bacterial contamination. The results in the mice were as follows:

Dried on glass thirty-one minutes	4D <sub>5</sub> , 4D <sub>6</sub> , 5D <sub>7</sub>
Dried on left hand seventeen minutes	7D <sub>5</sub> , 9D <sub>6</sub> , 9D <sub>7</sub>
Dried on right hand fifty minutes	9D <sub>5</sub> , 10K <sub>6</sub> , 10K <sub>7</sub>

#### DISCUSSION

Virus suspension made from the lungs of influenza mice used in the first two experiments seemed to be of rather low potency and to withstand drying very poorly. The lung virus used in Experiment 2 was perhaps slightly more active after drying on glass. This virus after drying on the palm of the hand also caused infection in all four inoculated mice, but of a milder type.

Experiment 3, in which allantoic virus was used, gave a strikingly different result in that all the inoculated mice died of the infection within five days and all with consolidation in all five pulmonary lobes. Here the virus evidently retained a high potency after being dry on the hand for ten minutes and after being dry on a glass surface for 200 minutes. In Experiment 4 the allantoic virus remained active forty minutes after it had been dried on the hand. In Experiment 5 the hand washed in the ordinary habitual way allowed the virus to retain its activity forty minutes after being dried on the skin. However, when soapy water was allowed to dry on the hands in Experiment 6, the virus subsequently dried on the same areas promptly lost its potency. An attempt to compare the inactivating effect of soapy skin with soap-free skin in Experiment 7 miscarried, possibly because of the low titer ( $10^{-2}$ ) of the virus used. Repetition with slightly more potent (titer  $10^{-3}$ ) allantoic virus in Experiment 8 showed inactivation of the virus on the soapy skin in ten minutes and persistence of some virus for thirty minutes on the soap-free skin. Experiment 9, in which the allantoic virus had a titer of  $10^{-5}$ , gave an even more clearly contrasted result, indicating loss of potency on the soapy skin and retained potency on the soap-free skin of the same person. The last three experiments 10, 11, and 12, show persistence of the virus on the hands washed in the ordinary manner and dried on a paper towel. Here the active virus could still be detected forty-five minutes and fifty minutes after drying had been completed.

Our results are somewhat at variance with those of Krueger and his associates. These observers used suspensions of mouse lung infected with influenza viruses of the P. R. 8 A strain and the Lee B strain. The hands were prepared by washing with soap and water and then held under a sterile towel to dry. Apparently special care to remove traces of soap was not exercised. Our observations were made with the Melbourne strain and for the most part

with virus-infected allantoic fluid, which seemed more potent than the suspensions of mouse lung which we used at first. Hence the technical methods were not identical.

The results of these drying experiments were somewhat variable and the variations cannot now be explained with entire satisfaction. However, the persistence of potency in an occasional specimen may be quite adequate to serve for transmission of the disease. We are therefore inclined to the view that soiled hands may reasonably be regarded as important for the spread of influenza, and especially the soiled hands of those concerned with the preparation and serving of food. Fortunately the virus of influenza appears to be readily inactivated by the ordinary soaps, a fact of importance to those who are concerned with the organization and inspection of establishments where food is served.

#### CONCLUSIONS

1. Potent influenza virus of the Melbourne strain may remain active on the palm of the human hand for forty-five minutes in the dry state.
2. The presence of soap solution tends to inactivate the virus on the skin.
3. Manual transmission of influenza, especially by food handlers, should be regarded as a possibility and a real menace.

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## GIANT ORTHOCHROMATIC ERYTHROBLASTS\*

### THEIR IMPORTANCE FOR THE PROMEGALORLAST AND PRONORMOBLAST PROBLEM

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EVER since Cohnheim<sup>1</sup> observed large nucleated and nonnucleated hemoglobiniferous cells in the bone marrow of a fatal case of Addison-Biermer's disease, students of morphology have advocated the specificity of these erythroid elements (megaloblasts and megalocytes) for this particular disease. With the advent of intravital bone marrow studies many investigators reported megaloblasts in the normal adult human bone marrow. Segerdahl,<sup>10</sup> Sabin,<sup>13</sup> and Doan<sup>3</sup> are leading proponents of the hypothesis that megaloblasts are constituents of the normal bone marrow. The first worker suggests that the reticulum serves as the source of origin of this immature erythroblast, which, according to her, is morphologically identical with the promegaloblast of Addisonian pernicious anemia, whereas the latter investigators derive the cell from the endothelium of "inter-sinusoidal capillaries." Introzzi<sup>7</sup> stresses the origin of the megaloblast directly from the reticulum. Mollier<sup>9</sup> and Knoll<sup>8</sup> showed that the erythroblast (early pronormoblast) may develop directly from the reticulum under certain conditions. Downey<sup>4</sup> believes that normally the myeloblast of Naegeli<sup>10</sup> gives rise to the pronormoblast. Jones<sup>6</sup> adheres to the hypothesis that the pronormoblast as well as the promegaloblast of Addisonian pernicious anemia is derived from the myeloblast. Nordenson<sup>11</sup> proposes that both the pronormoblast and the megaloblast are derivatives of the reticulum. Rohr<sup>12</sup> favors the origin of the erythroblast from undifferentiated mesenchymal cells, thus being in accord with Naegeli's hypothesis. Rohr points out that the very young proerythroblast (early pronormoblast) is difficult to separate from the myeloblast. Dameshek and Valentine<sup>2</sup> state that "cells resembling the more mature megaloblasts are occasionally seen in conditions other than pernicious anemia." It is likely that the latter investigators observed giant orthochromatic erythroblasts.

In my own collection of normal human bone marrow obtained by sternal puncture the earliest recognizable erythroid cell "early pronormoblast" has a round reticular nucleus with its chromatin arranged similar to that of the myeloblast. The chromatin, however, is more coarse, and the parachromatin is thus more conspicuous as in the latter cell. The nucleus contains from one to several fairly distinct light blue nucleoli. A more or less well-developed perinuclear "Hof" encloses diffuse, fine-to-coarse granular oxyphilic protein, easily seen when the bone marrow is prepared and stained according to a method published elsewhere.<sup>14</sup> The cytoplasm is not abundant, generally showing from one to several cytoplasmic processes, and it ranges in color from light to medium blue. This early erythroid cell is derived directly from the reticulum and is

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morphologically neither identical with the promegaloblast nor is the cell difficult to distinguish from the myeloblast.

It is absolutely essential that the proper name be applied to early erythroid cells, because the type of erythroblast present in the bone marrow determines therapeutic measures, and a loose application of terminology must obviously lead to misinterpretation of the mechanism of normal and pathologic erythropoiesis. For example, Schulten<sup>15</sup> believes that the pronormoblast develops into a large hemoglobiniferous erythroblast (megaloblast) because of a lack of the antipernicious factor (hematopoietin). According to Sabin, Doan, Segerdahl, and others, megaloblasts increase in number because of the disturbance in the production or utilization of the antipernicious factor. Megaloblastosis is thus the expression of a maturation arrest. It is felt by most students of morphology that the megaloblasts, or any one of their later maturation stages, are neither constituents of the normal bone marrow nor are these cells products of faulty differentiation of already well-differentiated cells such as the early or late pronormoblast.

Megaloblasts are pathologic cells and indicative of a specifically altered physicochemical state of the reticulum. The early promegaloblast is derived directly from a diseased reticulum, and this mode of origin accounts for the characteristic morphologic features of the cell.

Certain workers believe that it is not possible to separate the early promegaloblast from the early pronormoblast and thus have adopted the size of the oxyphilic erythroblast as the criterion for separating Addison-Biermer's disease from other entities. The size of an erythroblast does not determine the diagnosis in spite of the fact that in Addison-Biermer's disease oxyphilic erythroblasts generally range in size from 12 to 27 microns. That giant orthochromatic erythroblasts are not specific to the bone marrow of cases suffering from extreme exacerbation of Addison-Biermer's disease will be shown below. Two cases are presented in which the aspirated sternal bone marrow contained numerous giant orthochromatic erythroblasts, although their etiologic factor differed. Thus the fallacy of using the size of an orthochromatic erythroblast as a pathognomonic criterion in the diagnosis of Addison-Biermer's disease is apparent.

It is the purpose of this paper to show how two bone marrow lesions were misinterpreted and therapeutic measures misguided through (a) lack of appreciation of the type of early erythroblasts present, (b) misconception of the variability and nature of one of the lesions, and (c) misuse of the term "megaloblast."

If megaloblasts are part of a given bone marrow pattern, their presence means only one thing: "Addison-Biermer's disease" (Addisonian pernicious anemia), or a biologic variation of this complex hereditary disease. Only by adhering to the fundamental morphology of the early erythroblasts one may hope to learn something about the pathologic physiology of diseases affecting the bone marrow organ.

Case 1.—A male, 79 years old, complained of extreme weakness and pain in the back on bending over. With the exception of an unexplained anemia, physical findings were essentially negative. The peripheral blood status revealed hemoglobin 48 per cent; red blood cells, 2,300,000; white blood cells, 4,000; mean corpuscular diameter, 8.4 microns. His private

physician performed a sternal aspiration and submitted the bone marrow smears to a pathologist for interpretation. Because of the presence of numerous "large orthochromatic erythroblasts" in the bone marrow, the lesion was interpreted as characteristic of Addisonian pernicious anemia. The patient received 10 units of liver extract daily for twelve days without benefit. The dose of liver extract was changed to 30 units twice weekly for five weeks. Patient failed to respond and was hospitalized in a moribund condition. Sternal bone marrow was obtained, and a study of the peripheral blood was made on the day of admission.

The bone marrow preparations showed numerous "giant orthochromatic erythroblasts" (Fig. 1C), which exceeded the early and late pronormoblasts (Fig. 1d) in number, the ratio being about 4 to 1. The diameter of these giant erythroblasts ranged from 15.8 to 39.6 microns. The cells appeared to have a normal quota of hemoglobin. The nuclei were either lobulated, fragmented, or consisted of from five to eight deeply staining masses of irregular size. Some of the nuclear masses were connected by fine chromatin strands. In the majority of the cells the nuclear masses were eccentrically located as shown in Fig. 1C. These giant erythroblasts could be traced to the reticulum by means of many intermediate cells. No cells were observed that showed the morphology of the megaloblasts of Addison-Biermer's disease. Many monocytoïd reticulum cells were present either in small syncytia or as free cells which varied considerably in size. The morphologic patterns of the cells were distinctly pathologic. The bone marrow lesion was classified as belonging in the group of malignant lymphomata. Multipolar mitosis was not observed.

The peripheral blood showed: reticulocytes, 1.8 per cent; hemoglobin, 3.8 grams; red blood cells, 1,410,000; white blood cells, 3,200; hematocrit, 15 c.c. of packed red cells. For one hundred leucocytes there were eight oxyphilic erythroblasts ranging in size from 11.4 to 13.8 microns. Icteric index was 8 units. Gastric expression (alcohol-histamine test) showed no free hydrochloric acid. Examination of the bone marrow preparations made from the first sternal aspiration showed practically the same pattern as described above. Liver therapy thus did not alter the bone marrow lesion. Post-mortem examination revealed masses of various size in the mesentery. The tumors were made up of lymphoid tissue. Histologic diagnosis was "reticulum cell sarcoma."

Case 2.—A male, 82 years old, was admitted to the hospital in a moribund condition. Two months previously a sternal aspiration had been done by his private physician, and the bone marrow smears were submitted to a pathologist for interpretation. The striking feature in the preparations was the presence of a conspicuous number of very large "oxyphilic megaloblasts." Because the interpreter observed a lack of a diffuse basophilic megaloblastosis he believed the pattern to be the expression of some kind of malignant lesion (erythroleucemia). At that time the peripheral blood showed: reticulocytes, 0.3 per cent; hemoglobin, 5 grams; red blood cells, 1,560,000; white blood cells, 5,900. The blood smear showed a small number of erythroblasts in various stages of maturation. The patient received x-ray therapy over his slightly enlarged spleen. This treatment was reinforced by iron therapy. After the second x-ray treatment the patient failed to return to his physician. For three months previous to his hospitalization he was on a poor diet and without any kind of medication. On admission, a sternal aspiration was done. The preparations showed many "giant orthochromatic erythroblasts" (Fig. 1D) outnumbering the early and late basophilic erythroid cells in a ratio of about 3 to 1. The diameter of the giant erythroblasts ranged from 16 to 38.9 microns. The cells appeared to have a normal quota of hemoglobin. The majority of the giant cells showed a single round or oval nucleus. In others the nuclear mass consisted of three to seven more or less deeply staining nuclei eccentrically located. Thus the giant orthochromatic erythroblasts were morphologically identical to those observed in Case 1. An additional feature was the tendency toward multipolar mitosis in the early erythroblasts. Several giant orthochromatic erythroblasts showed mitotic figures. By means of many intermediate cells the giant orthochromatic erythroblasts could be traced to the reticulum. The early and late basophilic erythroid cells showed the characteristic nuclear pattern of the "megaloblasts" of Addison-Biermer's lesion of the bone marrow (Fig. 1B). In a recent paper by Jones,<sup>7</sup> the morphologic, physiologic, chemical, and biologic distinction of megaloblasts is reviewed in detail, and, therefore, a description of the megaloblasts is omitted. In addition, the myeloid cells showed the pathologic maturation pattern described

by Tempka and Braun<sup>17</sup> and Jones.<sup>7a</sup> The bone marrow lesion was classified as a unique variation of Addison-Biermer's disease. Examination of the peripheral blood showed: reticulocytes, 1.8 per cent; hemoglobin, 2.5 grams; red blood cells, 880,000; white blood cells, 1,900; mean corpuscular diameter, 9.5 microns; hematocrit, 12 c.c. of packed red cells; icteric index, 10. Gastric expression (histamine test) gave no free hydrochloric acid. The blood smear showed the characteristic pathology of the afore-mentioned disease. There were four oxyphilic erythroblasts with pyknotic nuclei among one hundred leucocytes. The erythroblasts ranged in size from 10.7 to 13.8 microns.

Patient expired a few hours after admission. Post-mortem examination revealed nothing remarkable. Weight of spleen was 240 grams. Final diagnosis was: advanced Addison-Biermer's disease (aplastic pernicious anemia).

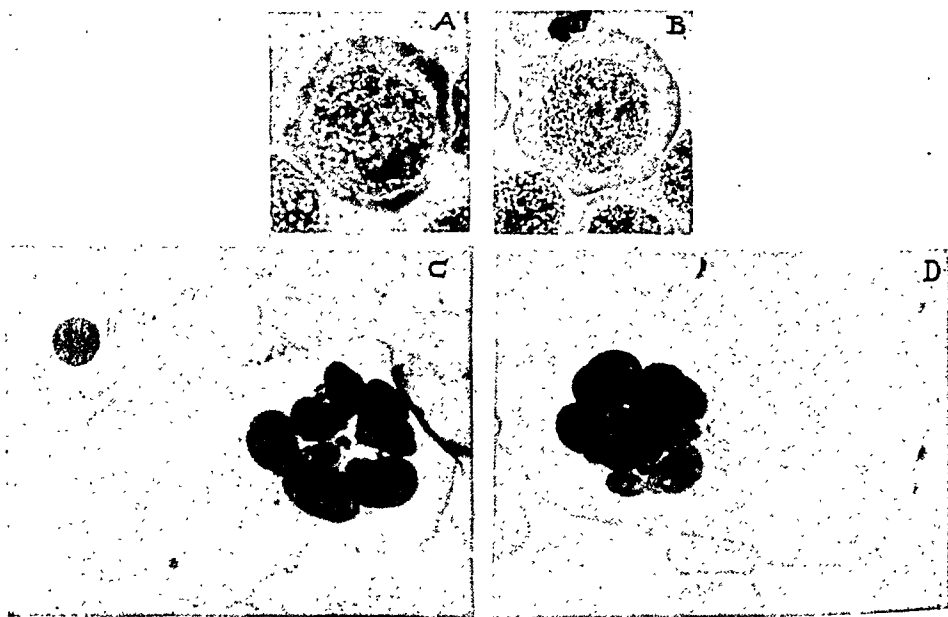


Fig. 1.—A, Early pronormoblast (Case 1). B, Early promegaloblast (Case 2). C, Giant orthochromatic erythroblast and orthochromatic macronormoblast in left upper corner (Case 1). D, Giant orthochromatic erythroblast (Case 2).

The histologic preparations of the post-mortem bone marrow showed several features of interest. There was a conspicuous number of Hedinger-Askanazy lymph follicles without germinal centers. Many of the collecting sinuses were markedly distended, thus giving an angiomatous appearance. The "Uferzellen" (reticulum cells lining the sinuses) were swollen and the nuclei were more or less pyknotic. Extensive reduction of hematogenic tissue and various degrees of sclerosis of the large- and medium-sized blood vessels as well as small areas of fibrosis, necrosis, and gelatinous transformation of fat were observed. In some of the sections made from material removed several centimeters away from the puncture, pools of blood were present, seemingly being the result of ruptured sinuses. Many megaloblastic islands were observed. Some tributary sinuses contained from a few to many polychromatic and orthochromatic giant erythroblasts, whereas the collecting sinuses contained only the small-sized oxyphilic erythroblast observed in the peripheral blood.

## DISCUSSION

Intravital bone marrow studies are becoming more and more important in clinical hematology. The value of such studies, however, is proportional to the discrimination of the finer structural differences of the nuclei of the immature, that is, early bone marrow cells, by the examiner. Failure to appreciate these differences has led to much confusion with respect to the type of erythropoiesis present in the normal and pathologic human bone marrow. While on the surface the promegaloblast, pronormoblast, and giant orthochromatic erythroblast problem appears to be of academic interest, yet the type of erythropoiesis present in the bone marrow determines the therapeutic measures.

Generally it is true that the orthochromatic megaloblast of Addison-Biermer's disease exceeds in size the definitive oxyphilic macronormoblast, but it could be shown that neither large nor giant orthochromatic erythroblasts can be used as a means of separating Addison-Biermer's disease from other clinical entities, because both types may be produced in the bone marrow by diseases other than that just cited. Furthermore, the assumption that the early and late basophilic megaloblasts in untreated cases of Addison-Biermer's lesion dominate the polychromatic and oxyphilic erythroblasts does not hold in all cases. Thus it is easily seen that neither criterion is sufficiently constant to be diagnostic. Examination of several thousand bone marrow preparations representing a large range of diseases affecting the bone marrow organ revealed the fact that the most reliable criterion of the type of erythropoiesis present is the nuclear pattern of the early erythroblasts.

A comparative study of the first and second bone marrow biopsy of Case 1 showed that the liver therapy did not appreciably alter the bone marrow pattern.\* Whether or not in Case 2 a timely administration of proper antianemic therapy would have had any beneficial effect on the pathologic state of the reticulum one may only guess. Attempted revitalization of a bone marrow that has undergone extreme physicochemical alteration complicated by irreversible lesions has met with failure in the few cases I have studied.

In both cases the early erythroblasts were derived from the reticulum, and, as can be clearly seen, each cell type shows a characteristic nucleus. The point of view of certain other investigators that the early promegaloblast and pronormoblast are not distinguishable is thus not valid. The giant orthochromatic erythroblasts were morphologically identical, but their precursors were different.

It is thus assumed that in either case the reticulum was differently affected because of the dissimilarity of the etiologic factor, leading on one hand to the production of pronormoblasts and, on the other, to the proliferation of promegaloblasts. It appears that a common mechanism was at work in the production of the giant orthochromatic erythroblasts, the nature of which, however, is conjectural. In none of the examined bone marrow preparations was I able to observe transitional stages between promegaloblasts and pronormoblasts, that is, the former giving rise or developing into the latter cells, or vice

\*Dr. Hal Downey has in his collection a bone marrow from a case of leucemic reticulo-endotheliosis that shows giant orthochromatic erythroblasts morphologically identical with those of Cases 1 and 2.

versa. This fact substantiates the opinion of those who believe that Addison-Biermer's disease affects the reticulum rather than the maturation of already well-differentiated cells.

It is assumed that normally reticulum cells go through an "inert" phase. During this latent stage one or more components of the hematopoietin exert their action upon the cells directing differentiation toward a specific cell type. Absence of one or more of these substances permits other factors to come into play, and such a faulty equilibrium between the bone marrow reticulum and the organs that control its normal function leads, for example, in Addison-Biermer's disease to the proliferation of promegaloblasts. The giant orthochromatic erythroblastosis is interpreted as the expression of a defunct reticulum.

#### SUMMARY

1. Two cases are reported which showed in the aspirated bone marrow numerous giant orthochromatic erythroblasts. Both cases were misdiagnosed because of a preponderance of these cells. Case 1 was afflicted with a reticulum cell sarcoma and Case 2 suffered from Addison-Biermer's disease (Addisonian pernicious anemia).

2. Giant orthochromatic erythroblastosis is not diagnostic of any specific disease. The phenomenon is the expression of a defunct reticulum.

3. The nuclear pattern of early erythroid cells, pronormoblasts and promegaloblasts respectively, is the only reliable means for judging the character of the erythropoiesis.

4. The early erythroblasts in either case were derived directly from the reticulum and showed characteristic nuclei.

5. The giant orthochromatic erythroblasts could be traced to the reticulum by means of intermediate cells. They were restricted to the bone marrow. Their morphology is described and illustrated.

I wish to thank Dr. G. E. Fahr, Head of the Department of Medicine, Minneapolis General Hospital, for his permission to publish the hematologic data of the cases presented here; Dr. Hal Downey for his kindness in permitting study of his case of leucemic reticulo-endotheliosis and for editing the manuscript; Dr. E. A. Sharp, Director of the Department of Clinical Investigation, Parke, Davis and Company, Detroit, Mich. for his interest in the anatomy, physiology, and pathology of the bone marrow organ. The photomicrographs were made by Mr. H. W. Morris, campus photographer.

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#### ADDENDUM

While this paper was in press, an article appeared in the *Arch. Path.* 36: 127, 1943, by Limarzi, L. R., and Levinson, S. A., in which the presence of giant orthochromatic erythroblasts in the aspirated sternal marrow was reported. The patient apparently had a cancerous lesion of the bone marrow organ.

## THE ANTIBACTERIAL EFFECTS OF VARIOUS ACRIDINE COMPOUNDS\*

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FOLLOWING the demonstration of the antiseptic value of the acridine derivatives by Browning and Gilmour<sup>1</sup> in 1914, several of these compounds, namely, acriflavine and proflavine, were successfully used during World War I in the treatment of wound infections. Since that time, however, interest in such compounds has gradually diminished, and until quite recently their use for the treatment of wound infections has been largely abandoned. This lack of interest during recent years is perhaps explainable, in part, by the impetus given to studies of sulfonamide derivatives by Domagk, who demonstrated that striking therapeutic effects may be obtained in systemic infections following medication with the various sulfa drugs.

Although the sulfonamides have proved to be of unquestionable benefit in chemotherapy of many systemic bacterial infections, they have certain drawbacks as agents for rapid and effective healing of surface wounds.<sup>2-5</sup> The members of the acridine group which show antiseptic activity in high dilutions against the majority of pathogenic bacteria are also nontoxic in these same dilutions to mammalian tissues and do not suppress phagocytosis.<sup>6</sup> Furthermore, their activity is unaffected by the presence of serum or organic matter.<sup>7</sup>

In a previous report<sup>8</sup> from these laboratories there was submitted evidence of the comparative in vitro effects of several acridines upon gram-negative bacteria of the colon-typhoid-dysentery group. The present communication presents data on the in vitro effects of these and additional acridines upon *Staphylococcus aureus* #209, beta hemolytic streptococcus #C-203, *Streptococcus viridans*, *Streptococcus agalactiae*, Types I, II, and III pneumococci, *Bacillus pyocyaneus*, *Clostridium welchii* and *Vibrion septique*.

The compounds studied, together with their corresponding pH values in distilled water and broth, as determined by the glass-electrode potentiometer, are listed in Table I.

### METHODS

A 1:1,000 dilution of each compound was prepared in a beef infusion 0.15 per cent dextrose broth of pH 7.4. A medium consisting of 1 per cent bacto-tryptone, 1 per cent neopeptone, and 0.15 per cent dextrose in distilled water and sealed under vaseline was used for the anaerobes. Serial dilutions through 1:512,000 were made in the same media. The tubes containing the drug dilutions and control broth tubes minus the drug were autoclaved at 10 pounds for 10 minutes. Each tube of a dilution series was then inoculated with a loopful of a 24-hour broth culture of one of the test organisms.

\*From the Research Laboratories, Winthrop Chemical Company, Inc.  
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TABLE I

NO.	COMPOUND	CHEMICAL FORMULA	pH OF 1:1,000 DILUTION	
			WATER	BROTH
1.	Acriflavine	3,6-diamino-acridine-10-methochloride-hydrochloride	5.0	7.4
2.	Proflavine	3,6-diamino-acridine-hydrochloride	2.5	7.4
3.	Rivanol lactate	2-ethoxy-6,9-diamino-acridine-lactate	5.6	7.4
4.	Rivanol-azo-sulfanilamide*	2-ethoxy-6,9-diamino-acridine-azo-sulfanilamide	4.5	7.4
5.	Rivanol-azo-sulfathiazole*	2-ethoxy-6,9-diamino-acridine-azo-sulfathiazole	3.5	7.4
6.	Atabrine dihydrochloride	3-chloro-7-methoxy-9-(1-methyl-4-diethylamino-butylamino)-acridine-dihydrochloride	5.3	7.4
7.	Atabrine dilactate	3-chloro-7-methoxy-9-(1-methyl-4-diethylamino-butylamino)-acridine-dilactate	6.2	7.4
8.	Atabrine dimethanesulfonate	3-chloro-7-methoxy-9-(1-methyl-4-diethylamino-butylamino)-acridine-dimethanesulfonate	5.6	7.4
9.	Acranil	3-chloro-7-methoxy-9-(2-hydroxy-3-diethylamino-propylamino)-acridine-dihydrochloride	5.2	7.4
10.	Neo-acranil	3-chloro-7-methoxy-9-(2-hydroxyethylamino-ethylamino)-acridine-dihydrochloride	5.1	7.4
11.	Entozon	2,3-dimethoxy-6-nitro-9-(2-hydroxy-3-diethylamino-propylamino)-acridine-dihydrochloride	4.4	7.4

\*The Rivanol-azo-sulfonamides were kindly supplied by Dr. Frederick Proescher, San Jose, California.

The tubes were incubated at 37° C. and examined for visible growth after 24, 48, and 72 hours. Lack of growth after 24 hours was considered evidence of bacteriostasis. Tubes which failed to show growth after 72 hours were tested for bactericidal activity by transferring 3 loopfuls of the drug-organism-broth mixture to a broth medium lacking the drug. Failure of growth to appear in the subculture tube was taken as evidence of bactericidal action on the part of the drug in the original medication tube. The results of these studies are presented in Tables II and III.

#### RESULTS

In summarizing the data given in the tables, we note that acriflavine, proflavine, rivanol lactate, and rivanol-azo-sulfanilamide give comparable bacteriostatic and bactericidal effects. Differences in the degree of effectiveness between any two of the above compounds upon the same test organism seldom involved more than two serial drug dilutions. Although the remaining acridines exhibited definite antibacterial properties, they were, in general, somewhat less effective than the first four compounds mentioned. The susceptibility of *Cl. welchii* and *V. septique* to the acridine compounds was of the same order as that of the gram-positive cocci. Much higher concentrations of the drugs were required, however, to inhibit the growth of *B. pyocyaneus*.

Unlike sulfonamide activity in vitro, which is generally recognized to be merely a bacteriostatic effect, the acridines used in this investigation exhibited a close correlation in the concentrations showing both bactericidal and bacteriostatic properties.

TABLE II  
HIGHEST DILUTION OF COMPOUND SHOWING ANTIBACTERIAL ACTIVITY

COMPOUND	PNEUMOCOCCUS						STREPTOCOCCUS					
	TYPE I		TYPE II		TYPE III		HEMOLYTIC		VIRIDANS		AGALACTIAE	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
Aeriflavine	128,000	128,000	256,000	32,000	128,000	64,000	256,000	128,000	64,000	32,000	256,000	256,000
Proflavine	256,000	128,000	256,000	128,000	128,000	128,000	256,000	128,000	128,000	64,000	128,000	128,000
Rivanol	64,000	64,000	128,000	128,000	128,000	128,000	128,000	64,000	64,000	64,000	128,000	64,000
Lactate												
Rivanol-azo-	128,000	64,000	256,000	128,000	64,000	32,000	128,000	64,000	64,000	64,000	128,000	64,000
Sulfanilamide												
Rivanol-azo-	16,000	16,000	64,000	32,000	8,000	4,000	16,000	16,000	8,000	4,000	16,000	8,000
Sulfathiazole												
Atabrine	128,000	128,000	128,000	128,000	64,000	64,000	64,000	32,000	16,000	4,000	16,000	16,000
Dihydrochloride												
Atabrine	32,000	32,000	32,000	16,000	4,000	4,000	2,000	2,000	8,000	8,000	4,000	4,000
Dilactate												
Atabrine	32,000	32,000	32,000	16,000	4,000	2,000	2,000	2,000	8,000	8,000	8,000	4,000
Dimethane-sulfonate												
Acranil	32,000	32,000	32,000	32,000	8,000	4,000	8,000	8,000	16,000	2,000	8,000	8,000
Neo-Acranil	32,000	16,000	32,000	32,000	8,000	4,000	16,000	16,000	16,000	2,000	8,000	8,000
Entozon	8,000	4,000	16,000	8,000	2,000	<1,000	16,000	8,000	4,000	2,000	16,000	8,000

Legend:—Bs = Bacteriostatic  
Bc = Bactericidal

< 1,000 = Dilutions less than 1:1,000 not tested.

TABLE III  
HIGHEST DILUTION OF COMPOUND SHOWING ANTIBACTERIAL ACTIVITY

COMPOUND	STAPHYLOCOCCUS AUREUS		BACILLUS PYOCYANEUS		CLOSTRIDIUM WEICHHI		VIBRION SEPTIQUE	
	Es*	Dc	Es	Dc	Es	Dc	Es	Dc
Acridiflavine	64,000	64,000	4,000	<1,000	256,000	8,000	256,000	128,000
Proflavine	64,000	16,000	4,000	<1,000	256,000	256,000	128,000	64,000
Rivanol Lactate	64,000	32,000	8,000	<1,000	256,000	4,000	128,000	64,000
Rivanol-azo-sulfanilamide	64,000	32,000	8,000	<1,000	64,000	32,000	64,000	16,000
Rivanol-azo-Sulfathiazole	8,000	4,000	2,000	<1,000	32,000	32,000	16,000	8,000
Atabrine Dihydrochloride	16,000	16,000	<1,000	<1,000	8,000	8,000	16,000	4,000
Atabrine Dilactate	8,000	4,000	<1,000	<1,000	32,000	16,000	8,000	4,000
Atabrine Dimethane-sulfonate	8,000	4,000	<1,000	<1,000	16,000	16,000	8,000	4,000
Acranil	16,000	8,000	<1,000	<1,000	16,000	8,000	2,000	2,000
Neo-acranil	16,000	4,000	<1,000	<1,000	2,000	2,000	2,000	2,000
Entozon	8,000	4,000	<1,000	<1,000	128,000	128,000	32,000	16,000

\*See footnote under Table II.

## CONCLUSIONS

Upon the basis of the experimental evidence presented in this report the following conclusions may be drawn.

1. Two acridine compounds, rivanol lactate and rivanol-azo-sulfanilamide, compare favorably in their antibacterial actions in vitro to acriflavine and proflavine.

2. Several atabrine salts and other acridine derivatives were found to exert definite bacteriostatic and bactericidal effects upon several of the organisms studied.

3. The mode of action of the acridines was found to be largely one of bacterial destruction rather than merely inhibition of growth.

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## GENERAL CONSIDERATIONS ON PATHOGENESIS: SYPHILITIC AORTITIS, MYOCARDITIS, HEPATIC CIRRHOSIS\*

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THIS paper presents a consideration of the possibility that allergic phenomena play a part in the development of certain diseases prevalent in Venezuela. Syphilitic aortitis, chronic myocarditis, and hepatic cirrhosis have certain clinical and pathologic features which provoke this discussion.

### SYPHILITIC AORTITIS

The cycles of manifestations of syphilis are well known. In successive stages, the lesions increase in destructiveness, and in the tertiary stages, when the patient is sensitized and allergic, this is especially conspicuous. In Venezuela, as in other regions, syphilitic aortitis is more frequent than are gummas in other organs.

Syphilitic lesions were found in about 17 per cent of all autopsies and were the cause of death in more than 50 per cent of this group. The ratio of males to females was 4.9 to 1 as compared with 1.7 to 1 in the nonsyphilitic patients. This may be due to greater disposition of women to follow courses of treatment faithfully. In 500 cases of syphilitic aortitis, that lesion was the only morphologic evidence of syphilis in most of the cases. In less than 10 per cent of these cases were other organs involved. This material is especially suitable for study, because intimal arteriosclerosis is rare among the people of Venezuela and seldom offers a complication of the picture. Marked arteriosclerosis is not frequent even in the aged. In contrast, syphilitic aortitis is common and is frequently observed in young people. Indeed it is not rare for death to be due to perforation of an aortic aneurysm in patients in the early twenties.

Topographically, syphilitic aortitis presents certain peculiarities. The site of predilection is the first part of the ascending aorta. The process sometimes involves only the ascending aorta, but may extend to the abdominal portion. Frequently, in contrast to the appearance as noted in the study of the aorta in Europe, the line of demarcation between the diseased and normal parts is not abrupt but fades gradually. In other cases, the condition is limited to the abdominal portion, occasionally involving only a single isolated area. The gross characters of the lesions are similar to those usually described: roughening of the intima, with irregular, stellate or longitudinal puckerings, and elevated, opaque, white, porcelain-like plaques.

The histologic picture in general is that usually seen. Cellular infiltrations are present in the adventitia, particularly around the vasa vasorum, and accompany these vessels into the media, where larger accumulations of cells and foci of necrosis of muscle and elastic fibers often occur.

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Entirely independent of these more familiar lesions are the microscopic changes in the intima. These consist of patchy plaques of relatively acellular homogeneous material along the intimal surface. These plaques are almost colorless in hematoxylin-eosin stain, whereas with the van Gieson technique they are pink or somewhat more red in later stages. At these sites the thick wavy band of the internal elastic membrane is missing, thus forming gaps in the membrane. No elastic fibers are detected in these intimal lesions, unless there be newly formed fibers in old healed cases. In some instances, there may be an indistinctness or complete loss of the elastic fibers in the inner media.

The question arises as to how such different lesions have developed. I believe with Benda that the infiltrations in the adventitia and media are gummatous, produced by the effects of the spirochetes in situ, the actual presence of which in such lesions has been demonstrated in several instances. This explanation is not applicable, however, to the lesions of the intima, since inflammatory features are absent. That the reaction is a compensatory hyperplasia of the tissue seems unlikely, inasmuch as such plaques have been observed in the absence of any defect in the media. Rather, it seems to me that these intimal thickenings as well as the alterations of the elastic membrane are the result of an allergic process. Similar changes in the aortic intima have been observed in other allergic conditions.

The sequence of development may be outlined briefly as follows: During the acute syphilitic infection, with widespread invasion of the human body with spirochetes and with progressive multiplication and death of the organisms, the body becomes sensitized. During the subsequent stages, with the reactivation of the infection, new spirochetes in minor numbers lodge here and there in the wall of the aorta and set up an allergic reaction in the tissue.

The opinion has at times been expressed that, since the use of arsphenamine in the treatment of syphilis, the frequency of syphilitic aortitis has increased. Local syphilologists believe that the great number of cases of syphilitic aortitis in Venezuela may be the result chiefly of inadequate treatment and that such treatment is more dangerous than no treatment at all.

These beliefs are tenable in the light of my explanation of the genesis of the lesions. During the treatment with arsphenamine many spirochetes are killed in a brief period of time and thereby the human body is definitely sensitized. Some spirochetes, however, remain alive, though dormant. When reactivated, they excite an allergic reaction, and produce the features described above. Syphilitic aortitis must then be regarded as the result of a combination of the reactions caused locally by the reactivated spirochetes themselves and of the allergic reactions.

#### CHRONIC MYOCARDITIS

The term "myocarditis" is used here to denote only the condition of actual inflammation of the myocardium. Myocarditis has been observed remarkably frequently in the material at my disposal in Venezuela. It has been diagnosed in more than 15 per cent of the autopsies and has been the cause of death in two-thirds of these cases. About 450 cases of myocarditis have been proved histologically.



Numerous cases have presented the typical clinical picture of progressively developing insufficiency of the heart, leading to death within a few months, although the earliest symptoms had appeared one or two years previously. In striking contrast, other cases have occurred in apparently healthy young persons who suddenly dropped dead at their work or on the street. Such extremes in variation in the course of the disease are impressive. It is difficult to understand how persons with such serious cardiac damage have remained symptom-free, only to die so suddenly. From a careful investigation of 165 such cases, it is concluded that there is a causal relation between the myocarditis and the sudden death. In fact, in my series of cases, myocarditis has proved to be second only to perforated aortic aneurysms as the cause of sudden death.

The anatomic picture in all these cases of myocarditis is so uniform both macroscopically and microscopically that no morphologic classification is possible. The hearts in general are more or less dilated, often enormously dilated, without displaying a corresponding degree of hypertrophy. The dilation may affect the entire heart uniformly or may be greater on one side or the other. Only rarely, actual dilation is lacking and the heart is merely flabby or relaxed. In such instances the myocardium appears dull and "parboiled" and sometimes is spotted or streaked with smaller or larger scars.

Histologically, the changes vary greatly as to sites of involvement and slightly as to degree and quality, dependent upon the intensity and duration of the process. The cellular infiltrations consist chiefly of lymphocytes, sometimes plasma cells and large mononuclear cells, frequently eosinophilic leucocytes, but rarely polymorphonuclear neutrophils. These areas of infiltration are more or less circumscribed but are not sharply demarcated from the surrounding normal tissue. They vary in size and numbers and tend to accumulate in the subepicardial and subendocardial regions. Cases in which the cellular infiltrations are limited entirely to these regions have not been included in the group of myocarditis.

In other instances collagenous fibrous connective tissue is increased locally in larger and smaller areas. In these regions a certain number of muscle fibers are missing and have been replaced by young connective tissue, in which the same cellular infiltrations are present. Finally, there are extensive large scars. The entire muscle tissue may at times appear edematous and the muscle fibers cloudy; rarely, there are focal areas of finely granular and fatty degeneration. On the other hand, in some of these cases, the muscle fibers show fatty degeneration and cloudy swelling without any alterations in the interstitial tissue.

The lesions described are not specific, in no way suggest the etiology, and permit no classification into various subgroups. An attempt was made to correlate the myocarditis with other diseases. It was found that in 44 per cent of the cases syphilis was also present; in 48 per cent there was schistosomiasis; and in 28 per cent there was ankylostomiasis. Thus in some, namely 28 per cent, there was more than one disease. In our total autopsy material, these diseases appeared in 17, 18, and 7 per cent respectively. Thus the association with myocarditis is not merely a matter of chance. There is probably a causative relation. About 10 per cent of the cases of myocarditis were not associated with any of these three diseases. Most of them accompanied sepsis or other infectious diseases.

In order to gain some understanding as to the uniformity of the changes in chronic myocarditis associated with syphilis, schistosomiasis, and ankylostomiasis, a brief consideration of myocarditis as it occurs in other geographic areas is worth while.

Myocarditis has been observed in various infectious diseases. In the majority of these instances the organisms produce local alterations in the tissue. In others, however, as in diphtheria, the presence of organisms in the myocardium cannot be proved and the alterations must be in response to the action of toxins. In a large group of cases of myocarditis, called by various terms such as "isolated myocarditis" or "Fiedler's myocarditis," the cause remains uncertain. Purely chemical poisons, as illuminating gas (with its carbon monoxide), and several sulfonamides (French and Weller) produce alterations in the myocardium similar grossly and microscopically to the forms above described. In some parasitic diseases, as trichiniasis, myocardial changes are frequent, even when the parasites cannot be found in the myocardium.

In the well-known books and reviews (e.g., Mönckeberg; Saphir), attempts are made to subdivide the myocarditides on an etiologic basis. However, the etiology seems so uncertain that generally the authors speak only of myocarditis in typhoid fever, myocarditis in diphtheria, and so forth, suggesting that they assume a causal relation but do not wish to convey the impression that the localization of the organisms must be the cause of the focal changes. A classification of myocarditis from purely morphologic viewpoints is impossible. Classification in relation to accompanying infections is valuable clinically but is unsatisfactory pathogenetically. In the light of genesis only two great groups of myocarditis can be differentiated:

1. Those in which the organisms are actually in the myocardium and produce tissue reactions by their presence. Such cases include myocarditis of septicemia, tuberculous myocarditis, gummas of the myocardium, and several other forms.

2. Those in which the myocardium is free from the organisms but is indirectly influenced by the organisms at distant sites, as for example in diphtheria.

I shall now consider more in detail the myocarditis of syphilis, schistosomiasis, and ankylostomiasis.

There is no doubt that there are cases of severe syphilitic myocarditis with typical gummas, in which the spirochetes are present in the cardiac muscle. The literature reveals extensive controversy as to whether nonspecific myocarditis is related to syphilis proper. Warthin maintained that in men with latent syphilis, alterations of the myocardium can always be found; in women, they occur less often. By means of a special method he was able to demonstrate the presence of spirochetes in nearly all such cases. This result, however, was obtained only by Warthin and his pupils. Most other investigators have reported negative results. Saphir expressed the opinion that the bodies considered by Warthin to be spirochetes were artifacts and that he himself had been unable to find spirochetes in any of 130 cases of myocarditis associated with syphilitic aortitis.

I believe that the frequent association of myocarditis with syphilis, as evident in the material here in Venezuela, cannot be coincidental. The facts that neither typical histologic syphilitic characteristics nor the spirochetes

themselves can be found in such cases indicate that local growth of the spirochetes is not the underlying factor, that the changes are in no sense specific, but not that the alterations have no syphilitic basis.

The alterations of myocarditis associated with infectious diseases, in which no organisms are found in the myocardium, may be caused by products of the organisms growing in other parts of the body. This opinion, however, seems to me not to be applicable in the case of the myocarditis in syphilis, or if applicable, only to a very limited extent. It is well known that in the secondary stage of syphilis many spirochetes are distributed throughout the body but that these tend to disappear until spirochetes are found only in small numbers or not at all. Alterations caused by diffusible products should, therefore, be apparent in the secondary stage. I have had no personal opportunity to examine hearts from the secondary stage nor have I been able to find reports of such studies in the literature. Certainly all my cases are of the tertiary stage, with anatomic lesions of tertiary syphilis, namely syphilitic aortitis. In such cases, circumscribed lesions actually exist; these are associated with the local presence of spirochetes though in very limited numbers. It seems difficult, therefore, to attribute the lesions of myocarditis solely to the effect of diffusible products from the remote spirochetes.

It may rather be assumed that during the secondary stage of syphilis the body has become sensitized to the spirochetes circulating in the blood. During the reactivation of the process in the tertiary stage, the spirochetes concentrated in small numbers in certain other parts of the body are analogous to the "determining" injection in an animal experiment. We deal here with an allergic process.

The features in bilharzial myocarditis are similar. That lesions in the heart muscle are produced locally by the presence of the parasites or their ova must be rejected. The parasites live practically exclusively in the portal venous system. I found ova in the heart only once in 200 cases of schistosomiasis showing myocarditis. It is very probable that the parasites themselves secrete injurious agents.

Most individuals suffering from schistosomiasis are infected repeatedly. Consequently, here too the possibility exists that the injurious substances act directly upon the heart muscle and that an allergic state also exists, with a corresponding flare-up in reaction produced by a new infection. I would assume that even in the first infection the heart muscle is damaged, a fact which cannot be proved anatomically, although in the routine examination of hearts from persons with schistosomiasis a certain number exhibit more or less extensive fatty degeneration without true myocarditis. Experimentally, my former pupil, Romero Reveron, was able to show that in infected guinea pigs, alterations that could be proved electrocardiographically exist in the hearts, although histologically there were only small cellular infiltrations. Such findings suggest that the myocardium has been affected from the first. It seems probable that in the acute stage of schistosomiasis, the heart muscle is injured by the diffusible products; with repeated infections an allergic component is added, thus producing serious alterations in the previously injured organ. Experiments with repeated infections in guinea pigs are now in progress.

Similar conditions exist frequently in ankylostomiasis. Infections may occur, are cured, and recur after variable periods of freedom from the disease. In severe infections, cardiac muscle may be the seat of marked fatty degeneration. Thus, there occur (1) repeated infections with intervening periods of cure or latency, and (2) local damage to the organ during the original infections. This means that the conditions for allergic reactions are found. As a result of the different degrees of severity of the infection and the corresponding injurious effects, varying periods of latency, treatment, and so forth, many variations in tissue reactions are conceivable.

A further example of the same principle is observed in the chronic myocarditis of Chagas' disease. I have had only a few opportunities to study this condition histologically, since it occurs only in limited districts of Venezuela. Mazza has described and illustrated a large number of similar cases from Argentina. From the apparent histologic similarities to my own few cases, it seems to me that it would be impossible to make the diagnosis without knowledge of the clinical features and the detection of the parasites.

It is striking that in the acute stage of Chagas' infection also, a great many parasites circulate in the blood, reaching all organs of the body, whereas in the chronic stage, with chronic myocarditis, very few parasites are found in the myocardium. The situation of the parasites does not always correspond to the site or extent of the lesions. The question often arises as to how so few parasites may cause such extensive effects. My explanation is that in the acute stage when many parasites are present, the individual becomes sensitized, so that in the later chronic stage, when few parasites are present, there is an allergic reaction to even a small number. Mazza objected to my explanation of the genesis of myocarditis in general and suggested that all the cases of myocarditis which I have observed in Venezuela are manifestations of Chagas' infection. The similarity of histologic picture in the forms herein described justifies an attempt at explanation on the basis of allergy, common to all.

To summarize, the changes in the myocardium may be placed in two groups:

Group I, comprising the cases in which the infecting organisms live in the myocardium itself and cause alterations in the heart muscle as they do in other organs and tissues of the body.

Group II, comprising the cases of myocarditis in which the presence of the organisms in the myocardium cannot be proved. Such alterations can be found in association with bacterial diseases, parasitic diseases, or with purely chemical poisonings and metabolic disturbances. Hence, this group may be divided into two subgroups:

A. Comprising the cases caused by pure poisonings.

B. Comprising the cases in which the alterations depend upon allergic tissue reaction.

Frequently it may happen that both types occur in combination. Parenchymatous alterations probably appear first, followed by further changes as a result of the altered metabolism of the parenchyma. Clinically, such different pictures as insidiously and progressively developing chronic illness and sudden unexpected death are observed. It must be supposed that in the first instance, repeated slight allergic reactions aid the development of the entire process; in

the second instance, anaphylactic shock kills the individual suddenly. It is also understandable that no significant differences in the hearts are found clinically or anatomically, no matter what disease or process has caused the myocardial alterations. The toxic and allergic reactions of the heart muscle remain the same, irrespective of the original disease, and thus the pathogenic process remains the same in all cases.

#### HEPATIC CIRRHOSIS

Cirrhosis of the liver affords a particularly striking example of failure in classification by the purely analytic method. Undoubtedly, every author engaged in a detailed study of the cirrhoses has tried to produce a satisfactory grouping of these processes. A division according to anatomic features is not possible, since there are no finally distinctive characteristics. Even the most simple and common differences between the hypertrophic and atrophic forms are not fundamental differences. The same histologic picture can be observed in both forms, and sometimes it is quite impossible for the pathologist with only a histologic section at hand to determine whether the cirrhosis is of the hypertrophic or atrophic type. In fact, it seems that whether hepatic cirrhosis is hypertrophic or atrophic depends upon accidental, environmental factors or upon the stages of the process. Furthermore, attempts have been made to classify the cirrhoses according to its associated manifestations, as splenomegaly, ascites, icterus, etc. Studies of cirrhosis in schistosomiasis have demonstrated, however, that even such manifestations are highly variable, and I agree with the authors who reject such a method of classification. Moreover, if a classification is based upon etiology, there are two groups, hepatic cirrhosis of known cause and hepatic cirrhosis of unknown cause. The majority of cases would belong to the second group. Such a classification would not take into account the pathologic features and the pathogenesis. Another possibility in grouping would be on the basis of damage to the parenchyma or to the connective tissue.

This fundamental problem I have previously discussed, as have many other authors. In 1920, I described by means of repeated biopsies the course of cirrhosis of the liver, experimentally induced. I expressed the opinion that every substance which in large doses injures the liver parenchyma, in small doses repeated over a period of time is capable of producing cirrhosis and that cirrhosis is caused only by the substances that have an injurious effect upon the liver parenchyma itself. Recently I was able to prove that in bilharzial cirrhosis, one of the few forms of which the actual cause is known, this principle holds good. In rare cases of this disease with probable acute massive infection, I could find evidences of damage to the parenchyma, even extensive necrosis. In the chronic stages there were all possible forms of cirrhosis. Therefore, I regard bilharzial cirrhosis in man as comparable to an experimentally produced cirrhosis. I came to the conclusion that cirrhosis of the liver develops as a result of injury to the liver cells, whether these injuries are visible microscopically or not. Then the metabolic products of the damaged liver cells influence the surrounding tissue and cause the further changes, which in themselves are dependent upon the type and severity of the primary damage to the parenchyma, and upon the general condition of the reacting tissue or individ-

ual. According to Josselin de Jong, a great variety of agents may cause a great variety of anatomic pictures. The etiology, the intensity of the damaging influence, the portal of entry, the duration of the process, the age, sex, and resistance of the individual in question, constitution, racial characteristics, climate, and other factors included under the term of "environment" may play a role. In a recent publication I said, therefore: "All these factors appear to be significant, not so much with regard to the form of the cirrhosis as to the type and intensity of the parenchymal damage upon which the further changes are dependent." Later in the present paper I shall discuss how these processes may be explained.

In summary, it must be concluded that the conception of cirrhosis should be uniform and I agree fully with Fiessinger, who said: "The anatomic process of the cirrhoses is the same in all the cirrhoses; there exists only one cirrhosis."

The process, which brings about the various pictures in cirrhosis of the liver, is after all essentially similar to that described for myocarditis. There is, however, one difference, inasmuch as the process in myocarditis is initiated by bacterial or parasitic products, whereas the process in cirrhosis in the majority of cases is probably initiated by disturbances in metabolism. It is especially interesting to observe that such different causes can lead to essentially the same process.

Before going further into the fundamental problem of the pathogenesis, it is well to emphasize that I find no significant difference between diseases that lead to a diffuse parenchymatous injury of the liver and the forms of diffuse hepatic cirrhosis that represent the result of parenchymatous injury. We recognize as diseases with purely parenchymatous disturbances: first, catarrhal jaundice, and second, acute yellow atrophy of the liver. We now know that catarrhal jaundice represents a parenchymatous disease and that it is not always a mild and harmless malady that heals in a brief time and that there are severe forms that may lead to acute yellow atrophy. On the other hand, we know that acute yellow atrophy is initiated almost always by a condition that cannot be distinguished from catarrhal jaundice. It is probably correct, therefore, to assume that these two pathologic pictures represent only the extremes of a single disease complex, and that between the two extremes all possible variations can be observed. In some cases there is healing only after a long time; in others death occurs after a shorter or longer duration; yet other individuals suffer from repeated attacks, a condition that is termed recurrent yellow atrophy of the liver. In some instances post-mortem examination reveals a more or less typical picture of cirrhosis of the liver. Our present knowledge does not permit us to distinguish these features grossly and histologically from true primary cirrhosis of the liver, because such differentiation is possible only in the light of the clinical course of the disease. Therefore, it seems entirely logical, particularly if the above-mentioned conception of cirrhosis is accepted, to consider these two forms as cirrhosis. It would be unreasonable to exclude them, as did Askanazy, for instance, because fundamentally they cannot be differentiated from the other types of cirrhosis.

In cirrhosis of the liver, a direct stimulation of the connective tissue does not exist; the changes in this tissue must be regarded as of a "regenerative or

reparative kind" (Rössle). The point emphasized by certain authors, for instance Rössle, that the reactions depend upon whether the damaging agents affect only the cells of the parenchyma or the connective tissue, Kupffer cells, or the capillary endothelium also, seems to me to be of minor importance. Injuries of this kind may be possible, although I have never observed them. They probably never occur without damage to the much more sensitive parenchymal cells. I believe, therefore, that I can justifiably present the following explanation of hepatic cirrhosis. It is a continuous process in which injury to the parenchymal cells is the first change. This stimulates regeneration and repair. Sometimes there is a compensatory regeneration of hepatic cells, but proliferation of connective tissue dominates the process. This explanation applies to all the conditions which belong in the broad field of hepatic cirrhosis and excludes from that category the purely toxic-degenerative, purely regenerative, and purely inflammatory lesions.

There is actually only one cirrhosis, and the various forms are caused mainly by variations in reaction. Such variations in reaction we denote today as allergy, and I believe that for the explanation of cirrhosis of the liver, allergic processes must be considered to a great extent.

Acute yellow atrophy of the liver is a sudden reaction of the entire organ to such an extreme degree that simultaneously an enormous number of cells die. Such a violent reaction could be explained only by acute and severe poisoning. It is known, however, that with only few exceptions, such as mushroom poisoning, no poison whatever can be detected. Experimentally, animals can be killed by agents that affect the parenchyma of the liver and serious alterations in the hepatic parenchyma can be produced without complete breakdown of the organ. In allergic processes violent reactions of the tissue occur, such as are seen in no other circumstances. Consequently, it seems logical to consider the process in acute yellow atrophy of the liver as an allergic reaction. Therefore, it is reasonable to believe that the liver has been affected by some previous damage and that with a new, even slight, injury an allergic reaction takes place in the previously diseased organ. If we start from this theoretical consideration, and if we accept the idea of the association between the damage of the parenchyma and the cirrhotic process, it would be logical to assume an allergic reaction also for the whole of the cirrhotic process. It then becomes clear that the reaction after a period of latency may be manifested in various forms, as a result of the intensity and duration of the injurious effect and may be dependent upon other external factors. Fundamentally, therefore, we must assume a damage to the liver parenchyma. Later, such damage may disappear or be reduced and allergic reactions may take place. Consequently, sometimes more toxic influences, sometimes more allergic reactions, and all possible combinations of these two factors may be the ultimate cause of lesions in the tissue. It is apparent that a great variety of possibilities leads to the diverse results and pathologic pictures.

These attempts at explanation are based upon hypothetical considerations. To me, however, these hypotheses have a logical foundation and facilitate a much better understanding of the cirrhotic process. Animal experiments were undertaken in 1930 to determine the validity of this hypothesis. Because of circumstances, these studies have not yet been completed, but give a clear

indication that allergic reactions play an important part in the development of acute injury to the hepatic parenchyma and the chronic lesions. Recently, Hartley and Lushbaugh came to similar conclusions.

#### SUMMARY AND CONCLUSIONS

An attempt has been made to show that often the analytic method cannot be employed to produce a really logical classification of diseases. In addition, it often fails to facilitate the understanding of pathogenesis. Clinical progress in diagnostic acumen and in therapy of diseases is possible only when the genesis of diseases is clear. For such understanding, I believe that synthesis is more important than analysis. We should not look for and describe the slightest variations in the alterations of the morphologic picture but we should rather search for the features in common, from which various deviations can be reasonably interpreted.

In studying the pathogenesis of various diseases, we must first know the etiology. Under etiology we must consider the extrinsic causative agent, as bacteria and parasites, or the intrinsic causative agent, as metabolic disturbances. However, different organisms and various disturbances in metabolism may both set up the same pathologic picture.

Equally important is the reaction of the body. It is a tenable assumption that the body may react similarly to various causes, especially if the etiologic agent affects the parenchymatous cells of organs and if these damaged cells produce further reactions as a result of faulty secretion and absorption of products of cellular disintegration. Furthermore, in many illnesses the pathologic processes may not be the result of a single transitory insult but often of a continuous or repeated insult. To these agents another important factor is added, namely, the allergic reaction of the individual, which in itself gives rise to the various tissue changes.

These considerations explain the fact that a single etiologic agent may produce diverse morphologic manifestations, and a variety of etiologic agents may operate to produce identical lesions. These deviations do not necessarily mean fundamental differences in the disease and separation into groups and subgroups, but simply indicate the manifold possibilities in the combined workings of the several factors.

To illustrate these principles, three entirely unrelated pathologic conditions have been considered:

1. In syphilitic aortitis, there is a combination of local tissue reaction to the organisms *in situ* and the allergic reaction.

2. Chronic myocarditis is to be regarded as a uniform process, in which there is primarily damage to the cardiac muscle fibers and, secondarily, mesenchymal alterations, with cellular infiltrations. This process, although purely inflammatory, must be distinguished from all myocardial inflammations in which organisms are present *in situ*. It is rather the allergic reaction of the cardiac muscle previously damaged. The myocarditis in syphilis, schistosomiasis, and ankylostomiasis have been shown to be of this type.

3. Although cirrhosis of the liver is caused by disturbances of metabolism in most instances, the same lesion can be produced by the injurious products of bilharzia. As a result of the findings in human beings and in animal experi-



ments, and by deduction, it is concluded that the process in cirrhosis of the liver is analogous to that in myocarditis; namely, that primarily there is parenchymatous damage and, secondarily, mesenchymal alteration. Cirrhosis, therefore, is the result of direct toxic effects combined with an allergic reaction.\*

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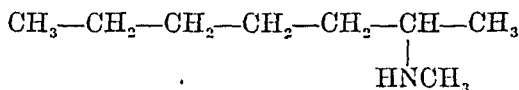
\*After having read Sulzberger's Introduction in the Form of a Series of Lectures, and that this author's ideas, as expressed in the last chapter, referring to the pathogenesis of syphilitic aortitis, tally with those presented in this paper.

\*Found great interest in reading Sulzberger's book "Introduction in the Form of a Series of Lectures, and that this author's ideas, as expressed in the last chapter, referring to the pathogenesis of syphilitic aortitis, tally with those presented in this paper."

## THE PHARMACOLOGIC ACTION OF 2-METHYLAMINOHEPTANE (EA-1)\*

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THE 2-methylaminoheptane is a clear, colorless, volatile fluid. It is only slightly soluble in water, but saturated aqueous solutions may be used for intravenous injections. The free base has the following formula:



Thus, the compound is an aliphatic amine, and it forms salts with a variety of acids of which I have generally used either the hydrochloride or the benzoate. Both are freely soluble in water, and a ten per cent solution has been used in the work described in this paper.† The hydrochloride and the sulfate are apparently not crystallizable salts, but the mucic acid salt is a fine white powder which lends itself readily to tablet manufacture.

In the experiments described herein this compound has been referred to as EA-1, followed by the designation of the salt used (HCl or benzoate as noted on the tracings). The EA-1 may be readily determined analytically by steam-distilling off the free base after previous addition of caustic soda to a sample and collecting the distillate in volumetric sulfuric acid and titrating back the excess acid with alkali, using methyl red as an indicator.

This compound belongs in the group of sympathomimetic amines, but its action differs from that of epinephrine in special features which will be discussed later. The EA-1 is a straight chain compound differing radically in its chemical structure from the usual sympathomimetic amines, such as epinephrine, ephedrine, neosynephrin, propadrine, benzedrine, paredrine, etc., all of which possess a benzene nucleus. Another straight chain compound which has been studied extensively is the nearly related 2-amino heptane,‡ which has been investigated especially by Proetz<sup>1</sup> and by Chen and their co-workers.

EA-1 produces notable symptoms when given by mouth in sufficient dosage; but the toxicity appears to be relatively low. For example, 3 c.c. of the 10 per cent hydrochloride solution (300 milligrams) given by means of a stomach tube to a dog weighing 24 pounds produced drooling of saliva, erection of a narrow streak of hair running in a central strip along the full length of the back, dilation of the pupils, slight lacrimation, rapid breathing, some depression, weakness, incoordination, and possibly some cardiac irregularity (missed beats?). Almost

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\*From the Department of Pharmacology, University of Cincinnati College of Medicine. Since this paper was written the name "amethine" has been given to this drug.

†I have obtained these preparations from the Bilhuber-Knoll Corp. of Orange, New Jersey.

‡This drug has been made available by Eli Lilly and Company in the form of a 1 per cent solution of the 2-aminoheptane sulfate under the trade name of "Tuamine Sulfate", which is used as a vasoconstrictor in the nasal passages.

complete recovery occurred in two and one-half hours, but the pupils were apparently still somewhat dilated for some hours longer. Possibly the drug may exercise a similar mild but *prolonged* dilator action on the bronchioles, since the innervation is similar in both cases.

When given intravenously, for example 1 c.c. (100 mg.) of the hydrochloride, in 10 per cent solution to a dog weighing 14.5 pounds, symptoms similar to those described above were produced, but they came on somewhat more promptly and involved a more extensive group of structures. The heart was affected (in the main accelerated), the pupils dilated, the bristles were raised, salivation and slight laceration occurred, and mild nervousness and tremors were produced. The pupils were rather widely dilated, and the light of a lamp bulb flashed into the eyes caused only a moderate constriction. This moderate loss of the light reflex is apparently due to persistent stimulation of the sympathetic nerve endings in the radiating fibers, and probably not to weakening or paralysis of the sphincter muscle. Tremors and muscular weakness were more marked when the drug was given intravenously, but a dog of this size (14.5 pounds) may make a fairly good recovery in two or three hours from an intravenous dose of twice this size (i.e., up to 200 milligrams). But the dilation of the pupils seems to be rather persistent and may indicate a usefulness of the drug in this relation.

In one dog, however, in contrast to the symptoms noted above, I have observed a course of symptoms which I believe is worthy of special attention. In this animal on each of four occasions when the drug was administered, either by mouth or intravenously, the symptoms began and progressed practically in the same manner as described above for about one-half to three-quarters of an hour, but then symptoms of an entirely different character began to appear. There was some indication of nausea, anxiety, crouching, or creeping movements, some disorientation or slight disturbance of consciousness, and then quickly a full-fledged convulsive attack occurred, with complete loss of consciousness, vomiting, dilation of the pupils, frothing at the mouth, and tetanic involvement of the whole skeletal musculature with opisthotonus and complete incoordination. With two or three brief remissions, the whole attack would last four or five minutes. The animal then relaxed, but appeared in a dazed or semiconscious condition; the breathing being deep and rapid (probably largely from the asphyxia produced by the convulsions). In another four or five minutes, however, the animal began to recover rapidly, and in a little while consciousness returned; the animal got up, walked around, responded to petting, and soon appeared to be entirely out of danger, but the pupils remained dilated and the breathing was rapid, drooling of saliva continued, and the bristles were raised. These latter symptoms disappeared in the course of two or three hours and the animal again appeared to become perfectly normal. In short, this dog appears to get full-fledged, grand mal epileptic attacks each time the drug is administered. Apparently this animal is a chronic epileptic, and the attacks which occur during the course of the drug's action are merely superimposed on, or perhaps sandwiched in between, the various phenomena produced by the drug. I have not yet had an opportunity to administer the drug to other known epileptic dogs, but it is my impression that the drug will probably have a similar action if given in

large doses to other epileptic animals. Perhaps this observation should serve as a precautionary warning in regard to using the drug with epileptic patients. I hardly think that this *evanescent* epileptic-like type of convulsive seizure is specific with this drug in normal animals within the dosage range indicated above.

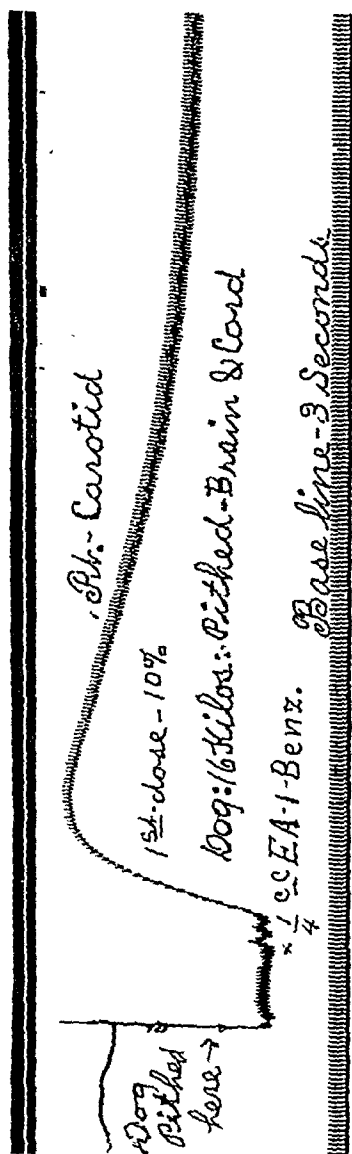


Fig. 1.—Carotid blood pressure in a dog with both brain and cord destroyed. At the left of the tracing the fall in blood pressure produced by pithing is shown. Following this  $\frac{1}{4}$  c.c. of EA-1 benzoate solution (10 per cent) was injected intravenously and the resulting rise in pressure is shown.

In general, within therapeutic limits, this drug appears to have but very little direct action on any portion of the normal central nervous system, unless there may be some slight feeling of weakness, lethargy, prostration, etc., which possibly may be central in origin. Nausea or vomiting apparently do not ordinarily occur under moderate dosage of the drug.

Fig. 1 shows the action of EA-1 benzoate on the blood pressure in a dog that had been completely pithed, both brain and cord. After destruction of the brain and medulla an iron rod was passed down the full length of the spinal canal and left in place throughout the experiment. Complete destruction of the cord was verified by the presence of the rod in the canal at autopsy. Care was taken to make certain of this point because of special differences between the action of this compound and that of adrenalin. So far as the action on the blood pressure and circulation is concerned, the drug apparently acts exactly the same in the pithed animal as it does in the intact animal. It can be seen from this tracing that the blood pressure rise comes on fairly promptly but is much more persistent and stable than is that which follows adrenalin. Here the EA-1 greatly improved the condition of the animal and permanently raised the blood pressure. I have repeatedly saved animals when they were in a very low condition and about to die by injecting just the right-sized (*first*) dose of this drug. Apparently most other drugs may still exercise approximately their normal actions after the first dose of EA-1 has been injected. But this does not hold in all cases, for the first dose of EA-1, or of the nearly related 2-aminoheptane, or of other active derivatives of the heptane series produces changes in the animal which markedly alter the effects produced by the second or any later injections of any one of these particular compounds. Even members of this series which have but little blood pressure raising activity may, if injected first, prevent a full rise which normally would have been produced by a following (first) injection of EA-1 or of 2-aminoheptane. This tachyphylactic action is frequently quite marked with these drugs, and may be of importance clinically in some organs, although the possible extent of this action is now obscure.

Fig. 2 shows the action of EA-1 hydrochloride after large quantities of nicotine had been injected and the autonomic ganglia had presumably been paralyzed. It seems probable in this case that the EA-1 was a little more effective in raising the blood pressure after nicotine than it would have been had the nicotine not been given. It would seem that some sort of synergism here exists between these two drugs. And I have seen a number of other instances in which I suspected that EA-1 became more active after other sympathomimetic drugs had been given shortly preceding. The manner in which such synergistic actions may be effected can only be surmised at present. From the evidence presented in Figs. 1 and 2, it would be presumed that EA-1 must act in the main on structures lying peripheral to the autonomic ganglia. I have not, however, been able to determine with complete certainty whether or not there may, in the normal intact animal, be some action by the drug on the autonomic ganglia. It is my opinion, however, that the drug does not have any significant ganglionic action.

Fig. 3 shows the action of adrenalin and of EA-1 after ergotoxine ethanesulfonate. The ergotoxine was dissolved in a small quantity of ethyl alcohol, which was then diluted with water and injected intravenously. It is difficult to get the ergotoxine into satisfactory solution because a rather high percentage of alcohol must be present to prevent precipitation. And when such a mixture is injected into the blood it may be expected that much of the ergotoxine will be dropped out of solution. I have tried to inject enough of the ergotoxine (in

many small repeated doses) to bring about the reversal of adrenalin action. The first injection of adrenalin in Fig. 3 shows a small preliminary rise in blood pressure followed by a fairly typical fall. The preliminary small rise, I believe, was due partly to the volume of fluid (5 or 6 c.c.) used to wash the drug

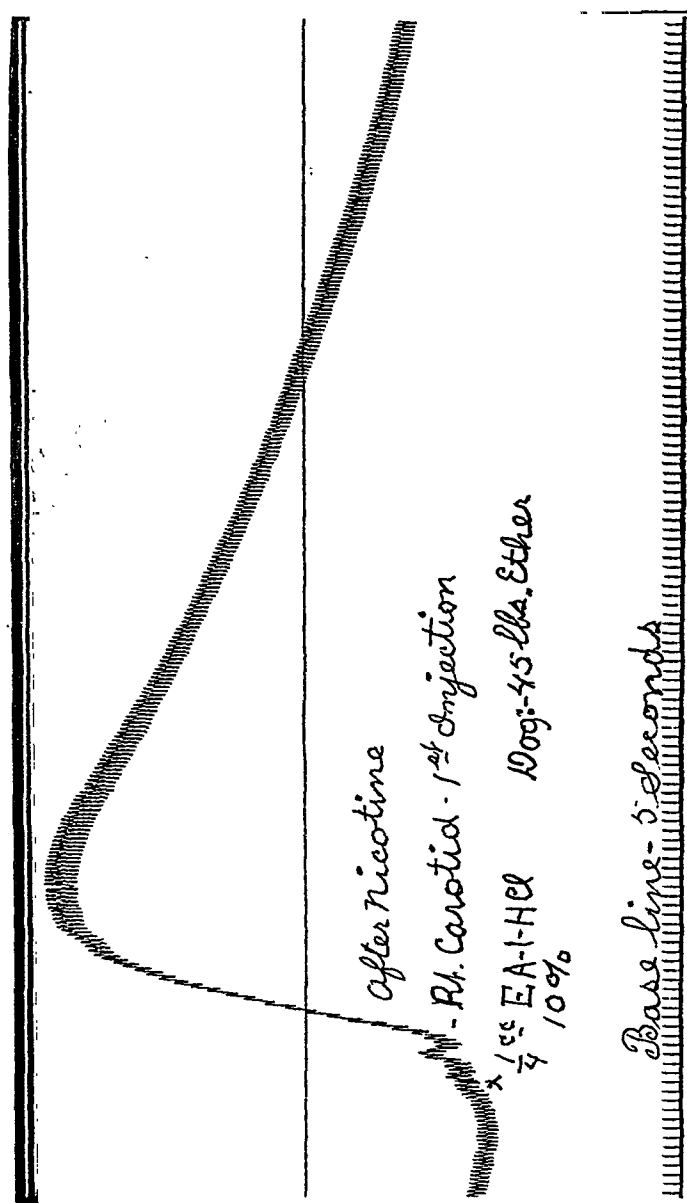


Fig. 2.—Carotid blood pressure tracing in a dog which had previously been given large quantities of nicotine by intravenous injection. The autonomic ganglia were presumably paralyzed by the nicotine.

into the vein and partly to a moderate acceleration of the heart beat from the action of the adrenalin. The curve of the fall and the recovery, however, seems to indicate that the vasoconstrictor endings no longer respond to stimulation by the adrenalin. Following this, an injection of  $\frac{1}{4}$  c.c. of EA-1 hydrochloride solution (10 per cent) was injected, and a prompt and extensive rise in blood



pressure was obtained. This shows that the action of this compound is different from that of adrenalin. And this point is further emphasized by the last injection ( $\frac{3}{8}$  c.c. of adrenalin) shown in the tracing. Here a very marked reversal action by the adrenalin is virtually superimposed on the rise in blood pressure caused by the EA-1. I have repeated this experiment in a good number of dogs, and there can be no doubt but that the results shown here are very accurate. There is one point involved in this experiment which I should like to emphasize. I have regularly found that after repeated doses of ergotoxine have been given, injections of adrenalin will usually still produce fairly marked rises in blood pressure followed by only moderate falls with fairly rapid rise back to normal. If at that time a single small dose of EA-1 be injected it is found that thereafter adrenalin will produce a perfectly typical, and generally very marked,

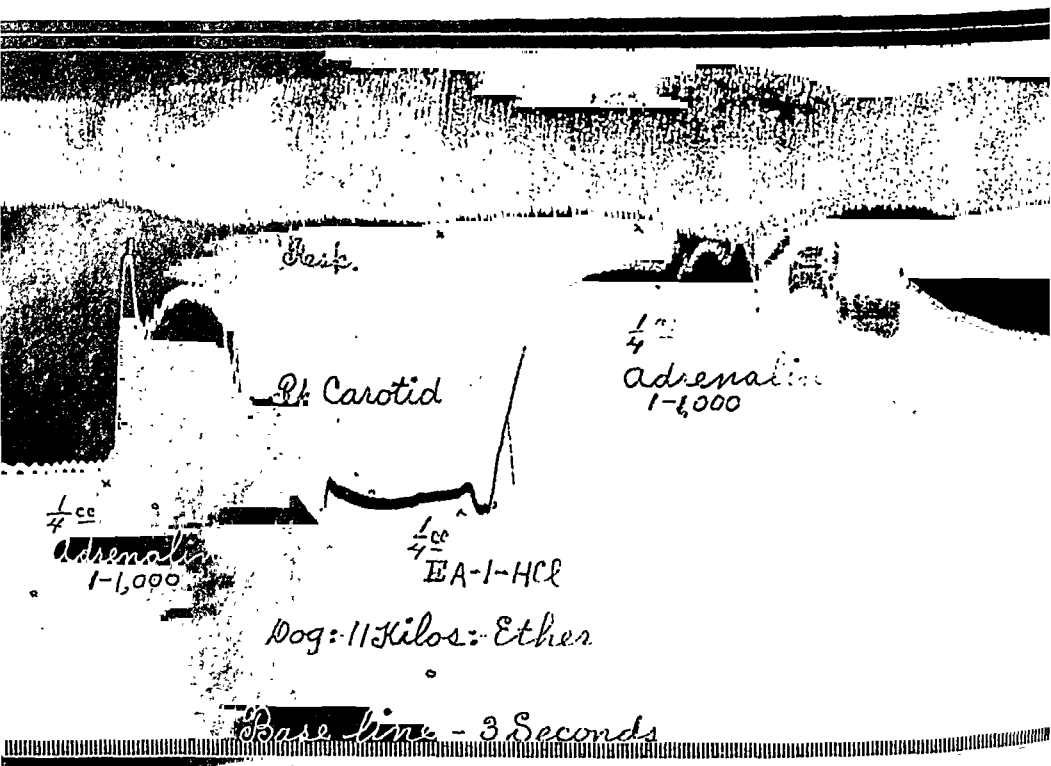


Fig. 4.—Respiration and blood pressure. This shows that the adrenalin action can be superimposed on that of EA-1. Note cardio-inhibition after adrenalin. For discussion, see text.

reversal fall each time the drug is injected. Thus the EA-1 appears to have some sort of synergistic action in the direction of making the reversal effects of the ergotoxine more prompt and emphatic, when adrenalin is injected later. Other sympathomimetic amines with which I have worked do not have this reversal action after ergotoxine and EA-1. But this phenomena led me to carry out the experiment illustrated in Fig. 4. In this experiment, as shown at the left of the tracing, a dose of adrenalin was injected and a typical adrenalin rise was produced. Following the return of the pressure to normal, a dose ( $\frac{1}{4}$  c.c.)



of EA-1 hydrochloride was injected. This produced a marked rise in pressure, at the highest part of which a second injection of adrenalin was given. The result is seen as a very obvious effort on the part of the circulatory system to raise the blood pressure still higher. The object of this experiment was to determine whether or not the EA-1 possessed any independent ergotoxine-like paralyzing action on the endings of the vasoconstrictor nerves. Apparently EA-1 merely acts synergistically with the ergotoxine but does not possess any independent reversing action of its own so far as adrenalin is concerned. I suspect this synergistic action with ergotoxine may be largely dependent on the cardiac action of EA-1 as illustrated in Fig. 5.

Fig. 5 shows the action of EA-1 on the heart and carotid blood pressure. Four separate injections were given, each  $\frac{1}{4}$  c.c. Here the marked stimulation and improvement of the heart by the first injection is well shown. But the first dose does something to the heart which becomes very obvious following each of the succeeding injections. There are certain features shown here which I should like to emphasize. The first dose produces a very marked improvement in the strength and amplitude of the heart beat. The blood pressure in this animal was low at the start, but there is a progressive and well-sustained rise even though each new injection reduces the amplitude of the heart beat. I suspect that this changed reaction of the heart after the primary injection is the reason why EA-1 appears to increase the reversal action of ergotoxine to following injections of adrenalin. The EA-1, I suspect, has a double action on the heart; first a stimulation of the sympathetic nerve endings, and, second, a direct muscular action which is, with proper dosage, almost entirely stimulating and augmentative, but which rapidly changes to depression with the second or later doses. Even if the first dose be a little too large, there is quite likely to be a preliminary fall in blood pressure before the general rise begins. This preliminary fall is apparently due to a direct cardiac action. If one wishes to utilize the drug to stimulate and restore an animal which is about to die, then it is very important to inject the right-sized dose at the beginning, and generally any further injections are likely to be more harmful than beneficial. I have repeatedly seen such striking results follow this use of the drug that I have been inclined to suspect that it might be useful in severe cases of shock or in other conditions in which the blood pressure is very low. In these cases, only one dose should be given and it should be of the optimum size. In a rough way, by comparison in animal experiments, I have found a dose of  $\frac{1}{4}$  c.c. of a 10 per cent solution of an EA-1 salt to equal approximately results produced by  $\frac{1}{4}$  c.c. of the one to a thousand solution of adrenalin chloride. But the effects of the latter drug wear off much quicker than do those of EA-1. In Fig. 1 this type of improvement is seen in a dog in which the entire central nervous system is destroyed (and a considerable hemorrhage had occurred). In Fig. 6 this action is perhaps more strikingly illustrated. In this case the animal at the start was about to die; then artificial respiration was provided and two large injections of adrenalin were given. But in spite of all this, the animal almost certainly would have died. But when EA-1 ( $\frac{1}{4}$  c.c.) was injected, there was a prompt and sustained recovery of the heart and blood pressure. While most sympathomimetic drugs are not likely to be of much lasting help in cases of severe shock, yet I have rather strongly suspected that EA-1

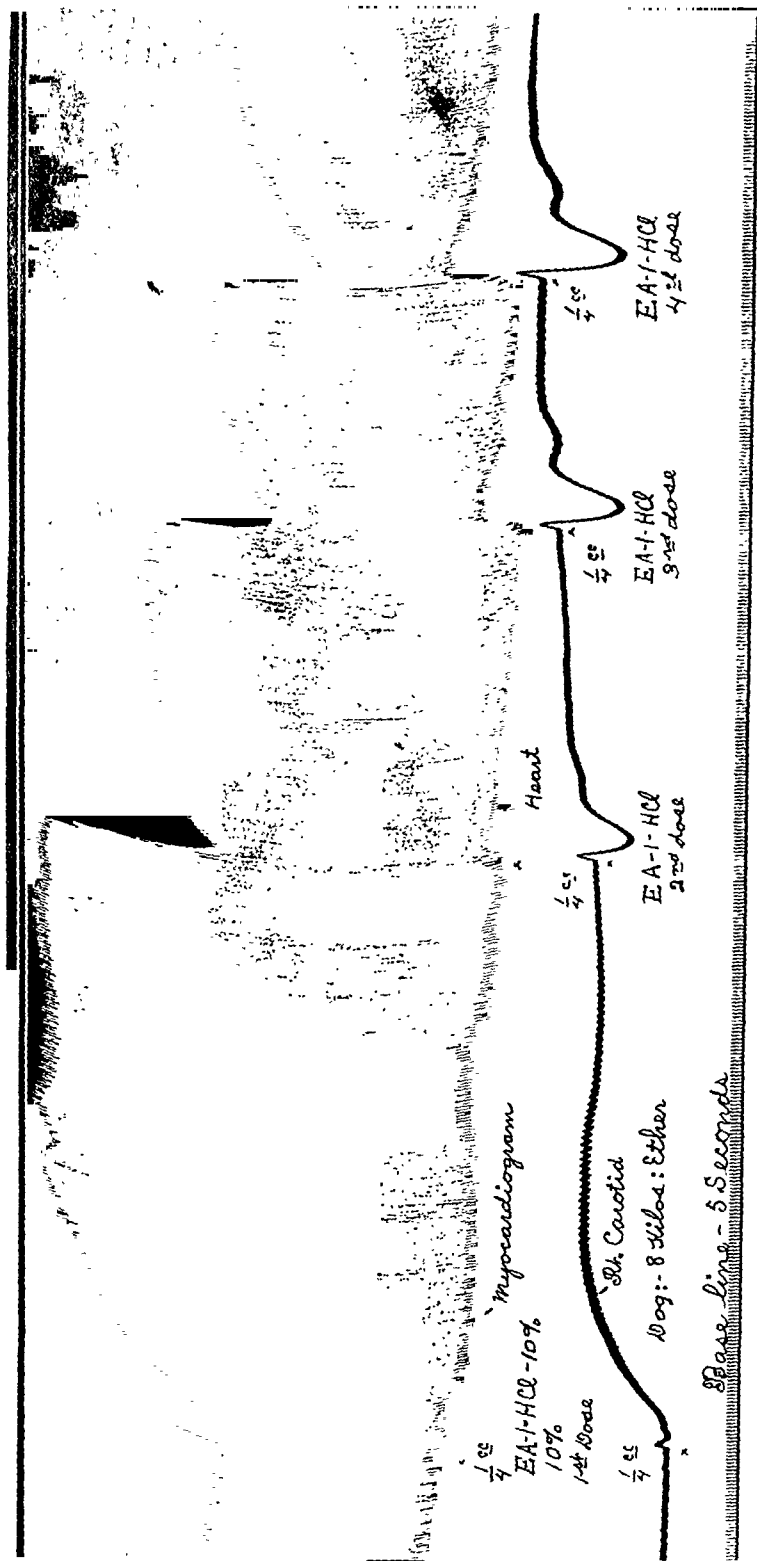


Fig. 5.—Myocardiogram and carotid blood pressure showing the results of four injections of EA-1. Note progressive rise in blood pressure.



Fig. 6.—Carotid blood pressure in an animal near death. Showing comparative actions of adrenalin and EA-1. For discussion, see text.

properly administered intravenously might often tide a patient over until he could be brought to a place where more extensive treatment could be applied.

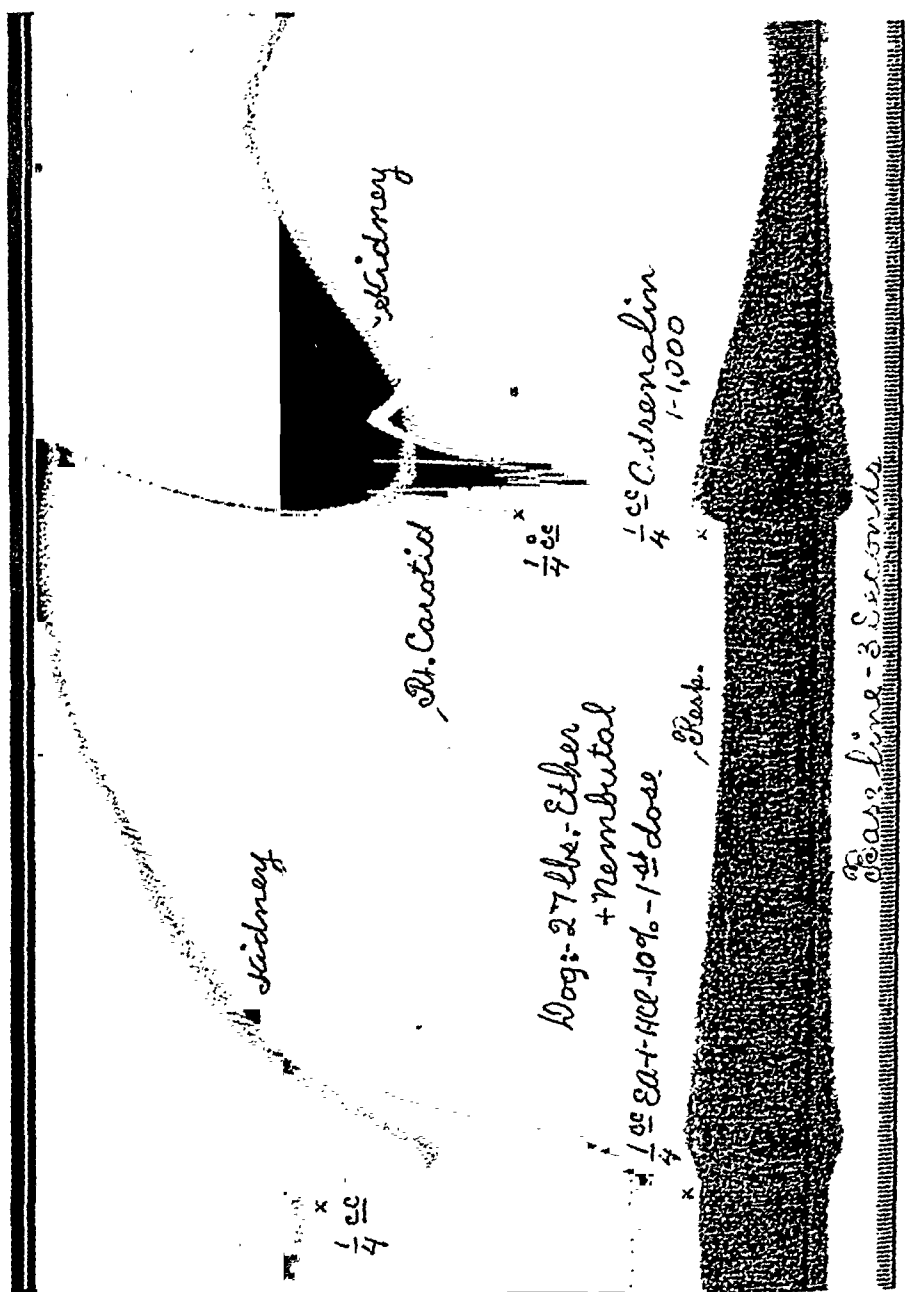


FIG. 7.—Kidney volume, blood pressure, and respiration showing the comparative actions of EA-1 and adrenalin. Note absence of cardio-inhibition after EA-1 and the marked inhibition after adrenalin.

One may readily see this action in an animal in which the brain, medulla, and upper part of the cord have been destroyed. And I have considered the possibility that this drug, if properly administered, might be used in some respects as a kind of substitute for digitalis. With moderate doses I have not seen as

much tendency to cardiac irregularities with EA-1 as would probably have occurred with comparable doses of the other sympathomimetic drugs with which I have worked. I have not made electrocardiographic records.

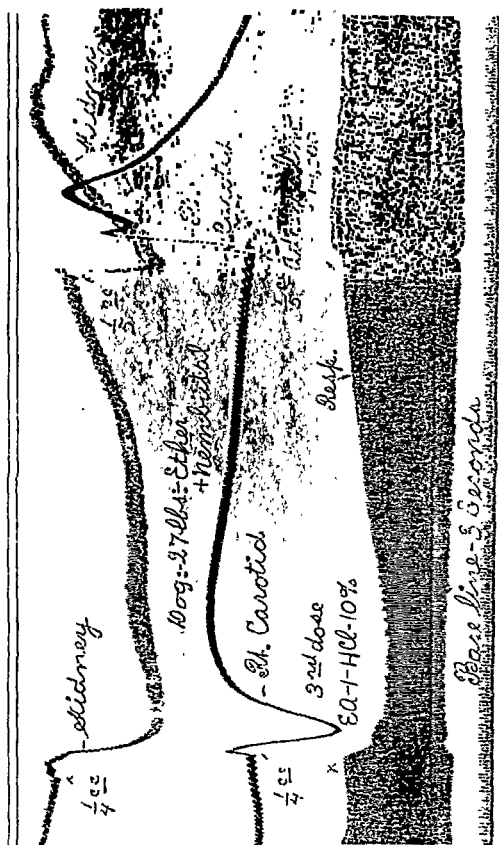


Fig. 8.—Kidney volume, blood pressure, and respiration showing comparative late effects of EA-1 and adrenalin.

Figs. 7 and 8 were both made in the same experiment. They show the results of the first and third injections of EA-1 together with injections of adrenalin for comparison. The action of EA-1 on the kidney is seen as a marked shrinkage in volume due to vasoconstriction. The vessels contract down to fully as small a caliber under EA-1 as they do under adrenalin, but the following dilation may vary somewhat in different experiments. Since the carotid

pressure in Fig. 7 rises much higher after EA-1 (the first dose) than it does in Fig. 8 (after the third dose) in which there is a considerable preliminary fall in pressure, one would expect that the kidney vessel constriction should be greater in Fig. 7 than in Fig. 8. This varies a good deal in different experiments, but in general it appears that the peripheral vascular constriction tends to become a little more intense with each repeated injection. This constriction is independent of the primary fall in pressure which tends to become more marked with each succeeding injection and which appears to be mainly due to the action on the heart. The vasoconstriction which occurs in the kidney is typical apparently of that which occurs in the other organs. The question might naturally arise as to what action EA-1 has on the arterioles of the pulmonary circulation, since these are in a somewhat different category from the systemic arterioles. Fig. 9 shows the action of EA-1 on the pulmonary blood pressure, which rises from fifty to one hundred per cent above the normal and tends to remain at this high level. The drug seems to have a very definite action here, and I believe that this is mainly due to stimulation and strengthening of the heart. There may be a specific constriction action on the pulmonary arterioles, but I believe that increased strength of the contractions of the right ventricle is the chief factor concerned. Fig. 9 also shows a profound constriction of the vessels in the nasal walls. It is to be noted that the drug here was injected into the femoral vein, but the vessels of the nasal walls and of the adjacent sinus membranes contract promptly just as do those of the kidney and other visceral organs. This is the usual action of sympathomimetic drugs, and it indicates a useful service which EA-1 may render in overcoming nasal congestion. The volatile free base may be inhaled for this purpose, or solutions of the salts may be applied locally. The nasal record (labeled 'nose') was made by closing off (airtight) the nasopharynx by means of a sponge rubber ball on the end of a rod, the ball being pressed against the soft palate which was thus forced backward against the posterior nasopharyngeal wall. Two glass cannulas were inserted (airtight) into the nostrils, and the cannulas were connected by means of a Y-tube and rubber tubing to a tambour. Shrinkage of the nasal walls (enlargement of the cavity) drew air out of the tambour, and the writing point moved downward on the drum.

Fig. 10 shows the action of EA-1 on the bronchioles. The animal was pithed and air was intermittently aspirated out of the chest in the usual manner with a plethysmograph placed on the chest. At the beginning of the tracing two injections ( $\frac{1}{2}$  c.c. and 1 c.c.) of eserine solution were injected into the femoral vein in order to bring on a bronchoconstriction. When this became well marked, a dose ( $\frac{1}{4}$  c.c.) of EA-1 solution was injected. This caused a bronchodilation of moderate degree. A later dose of EA-1 did not produce any further dilatation. The effect of the EA-1 here is probably less than would have been produced by adrenalin. But the action may be more prolonged. There is also some likelihood that EA-1 may be effective on the bronchioles when taken by mouth. The action apparently depends on stimulation of the bronchodilator nerve endings. In passing, it might be noted here that electrical stimulation of the vagus nerves in the neck causes bronchoconstriction after EA-1 has been administered and also the heart is simultaneously inhibited, so that EA-1 does not paralyze

the ganglia. It might be noted in Fig. 10 that the blood pressure was very low (brain and medulla and upper cord destroyed) at the beginning of the record. But two doses of EA-1 brought the pressure up and maintained it at very nearly the normal level as it had existed before the animal was pithed. It would be rare that a patient would sustain a more severe injury than that involved in total destruction of the brain and upper cord (with the chest widely opened besides), so that the results produced here on the circulation might indicate some usefulness of the drug in severe traumatic shock.

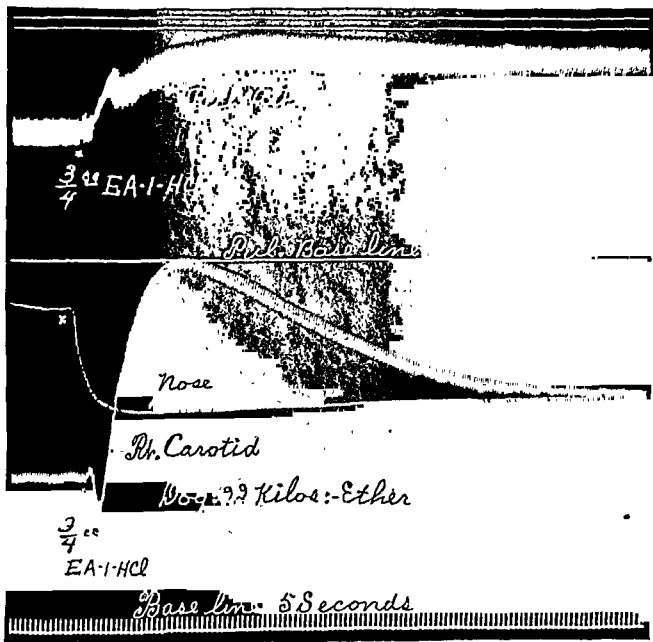


Fig. 9.—Pulmonary blood pressure, nasal cavity volume ("nose") and carotid blood pressure showing the action of EA-1. Note absence of cardiac inhibition. For discussion, see text.

In connection with the respiration, it might be noted that in anesthetized animals large doses of EA-1 cause death by paralysis of the respiratory center. But there is seldom seen any respiratory stimulation in animals anesthetized with ether or nembutal.

The usual action on the stomach in anesthetized animals is shown in Fig. 11. Some relaxation and inhibition of tone is obvious, but the action is less pronounced than that produced by adrenalin as shown in the right hand portion of the tracing. But the action of EA-1 is more lasting, and in addition it probably





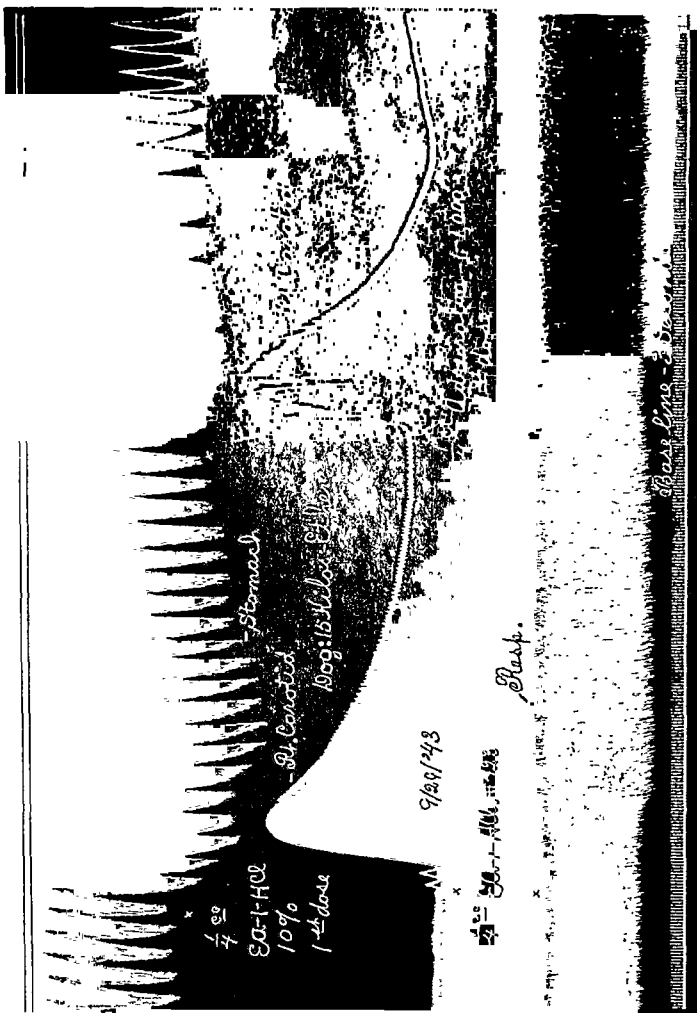


Fig. 11.—Stomach tracing, blood pressure, and respiration. Showing the comparative actions of EA-1 and adrenalin. Note cardiac inhibition after adrenalin but not after EA-1. The pressure elevation was approximately the same in each case. See text.

can be elicited by oral administration of the drug. Also the intestines are not much affected by EA-1 in acute experiments. It seems that the inhibitory action of the drug is, in general, considerably less marked than is the motor action on the circulation. And the bladder is also but little, if any, affected. It is difficult to surmise what kind of clinical effects might be obtained by an action on the stomach such as that shown in Fig. 11. Would such a relaxation in tone bring relief from colicky pains even though the peristaltic movements continued?

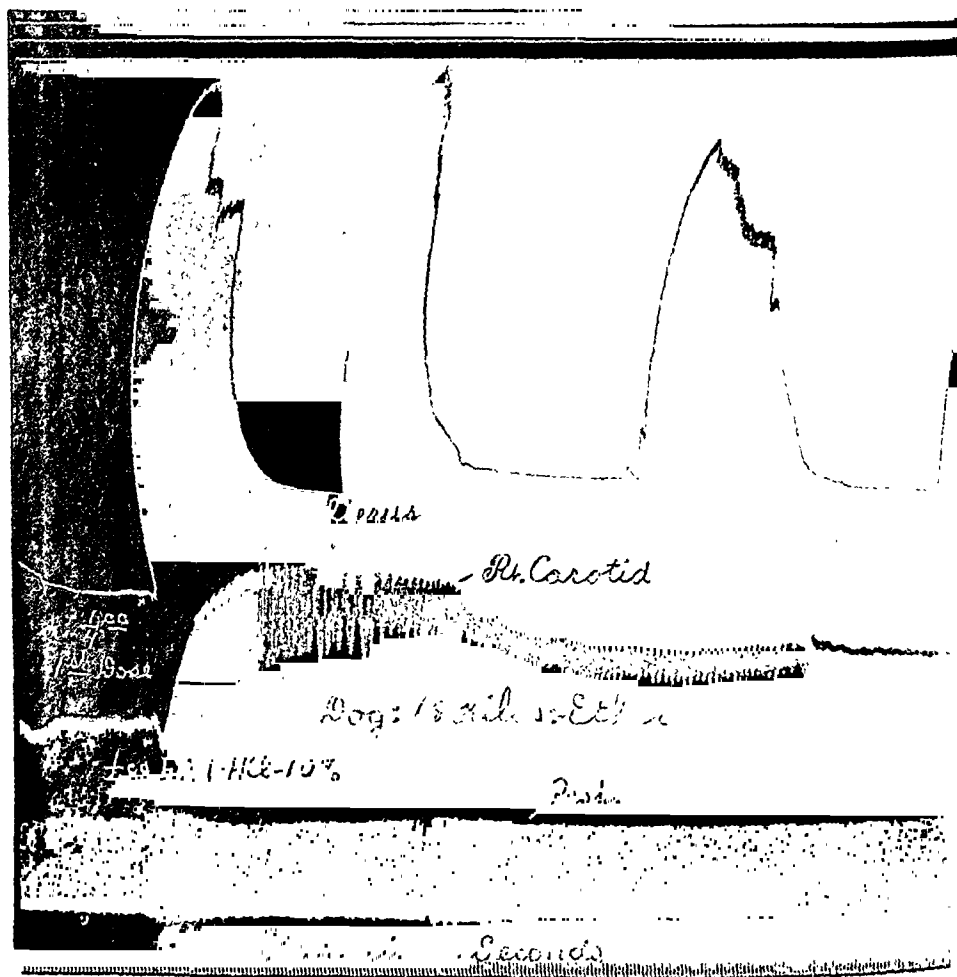


Fig. 12.—Uterus tracing, blood pressure, and respiration in an animal near term. Note cardiac inhibition (rare) in this case after EA-1. For discussion, see text.

It seems that EA-1 has but little, if any, action on the uterus. Fig. 12 shows a series of vigorous uterine contractions in an animal near term. The injection of EA-1 produced a marked action on the circulation but so far as the uterus is concerned only one thing can be said, viz., that the contractions were not inhibited. There may possibly be some slight increase in the general tone level of the uterine tracing (intact animal). But the marked intermittent con-

tractions shown in the tracing were occurring spontaneously before the drug was injected, so that the only conclusion permissible is that EA-1 does not inhibit the contractions. That point might be significant in case the drug should be used during labor. In other experiments in intact animals I have failed to note any special action of any kind on the uterus.

I have presumed that the chief action of this drug on the circulation involves primarily the heart and arterioles. But the capillaries may also be especially involved, by an action somewhat the reverse of that of histamine. The drug bears some similarity to ergotoxine in its action, and this has led me to wonder if it might be useful in migraine. I hardly believe that its continued use would lead to gangrene. I have no suggestions regarding any relation which the action of this drug may have to adrenergic, cholinergic, esterase, hormonal, etc., phenomena. But it would be very interesting to study this compound in accordance with the splendid method of clinical assay of sympathomimetic drugs which has recently been so well developed by Vaughan, Perkins, and Derbes.<sup>2</sup>

A consideration of the action of a compound such as EA-1 calls to mind such subjects as asthma, angioneurotic edema, anaphylactic shock, urticaria, ciliary activity, general allergic conditions, nasal decongestion, local vasoconstriction on hypodermic injection, sympathetic secretions, traumatic shock, pupillary dilatation, cardiac stimulation, serum sickness, development of tolerance for the drug, etc.

#### SUMMARY

The general pharmacologic action of a special sympathomimetic drug, 2-methylaminoheptane (EA-1), is discussed and illustrated by tracings.

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## SIMPLIFICATION OF THE TREATMENT OF DIABETES\*

CAPT. LAZARUS L. PENNOCK, M.C., U. S. ARMY

TODAY, more than twenty years after the introduction of insulin in the treatment of diabetes, the prescribing of diabetic diets is still in a state of mild confusion. It seems that every writer on diabetes has his own method for writing a diet. As a result, many methods for writing diabetic diets have been described. Some use complicated surface area tables. Others use set standard formulas, four to six of them, for all diabetic patients. Even a slide rule has been designed, to calculate a diabetic diet, by a drug firm. And in competition another firm has brought forth a "simpler" mechanical aid for the same purpose.

All this has made for confusion among the average practitioners of medicine who see a few diabetic patients now and then. In despair, most of the diabetic patients are referred to a specialist. A simple but satisfactory formula for computing a diabetic diet has been a necessity for some time. Such a diet should be individualized, tailor-made for each patient, standardized according to physiologic principles to meet the needs of each patient. And it should be properly balanced according to the best of our knowledge of nutrition today.

The essential thing to be borne in mind is that the diabetic diet should be a physiologic one, based on known values of the normal diet. The average individual, left to himself, chooses a diet that is not physiologic. He eats too much and the components of his diet are not proportioned in accord with what has been proved to be ideal for optimum nutrition. This is the chief fallacy of the so-called "free diet" treatment of diabetes.

The dietary treatment of diabetes is no longer, and in point of fact never was, a question of high carbohydrate versus high fat diet. Since a physiologic diet is to be prescribed, examination of such a diet reveals that the total daily caloric allowance is divided into carbohydrate, 65 per cent; protein, 15 per cent; and fat, 20 per cent. Such an arrangement always yields a diet that is high in carbohydrate and proportionately lower in fat. Hence, the diabetic diet should approach these proportions as nearly as possible, and the method of calculating a diet that will be described here does just that in a very simple manner.

In addition, the diet is calculated for the patient's normal or physiologic weight. Such weights are derived from the actuarial tables for age-height-weight, one of which is presented herein and which may also be found in almost any book on diabetes or nutrition.

These facts constitute the background for the method to be described. The method has been in use since 1924, when it was first propounded by Barach. For the past eleven years I have not only seen it applied, but have used it myself and watched it work successfully in all types of diabetic pa-

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tients. It never fails if one keeps in mind that, once the diet has been prescribed, the patient should be maintained on it until his metabolism has become adjusted to the diet. This will invariably come about in the course of a few days. Then, if the patient still continues to show sugar, insulin is given in sufficient amounts to control the hyperglycemia and glycosuria without changing the diet as a whole.

There are two essentials in calculating a maintenance diabetic diet. They are (1) determining the total daily caloric allowance per day for the patient and (2) dividing this allowance into the proper proportions of carbohydrate, protein and fat.

#### STEPS IN CALCULATING A MAINTENANCE DIET

1. Determine normal body weight in pounds for age and height of the patient (Table I). Convert pounds to kilograms by dividing by 2.2 (Table II).
2. Determine the caloric allowance per kilogram normal body weight.
3. Multiply kilograms normal weight by caloric allowance per kilogram for total daily caloric allowance.
4. Divide the caloric allowance into carbohydrate, protein, and fat.

TABLE I  
NORMAL WEIGHT—17 TO 34 YEARS

AGES	17—19	20—24	25—29	30—34
HEIGHT	M—F	M—F	M—F	M—F
4' 10"	109—1	115—4	120—6	123—6
4' 11"	111—1	117—4	122—6	125—6
5' 0"	113—1	119—4	124—6	127—6
5' 1"	115—1	121—4	126—6	129—6
5' 2"	118—1	124—4	128—6	131—6
5' 3"	121—1	127—4	131—5	134—6
5' 4"	124—1	131—5	134—5	137—5
5' 5"	128—2	135—6	138—6	141—5
5' 6"	132—2	139—6	142—6	145—5
5' 7"	136—2	142—5	146—6	149—5
5' 8"	140—2	146—5	150—6	154—6
5' 9"	144—3	150—5	154—6	158—6
5' 10"	148—3	154—5	158—6	163—8
5' 11"	153—3	158—5	163—8	168—10
6' 0"	158—3	163—6	169—10	174—12
6' 1"	163—3	168—6	175—12	180—14

NORMAL WEIGHT—34 TO 54 YEARS

AGES	35—39	40—44	45—49	50—54
HEIGHT	M—F	M—F	M—F	M—F
4' 10"	125—5	128—4	130—3	131—2
4' 11"	127—5	130—4	132—3	133—2
5' 0"	129—5	132—4	134—3	135—2
5' 1"	131—5	134—4	136—3	137—2
5' 2"	133—4	136—3	138—2	139—1
5' 3"	136—4	139—3	141—2	142—1
5' 4"	140—4	142—3	144—2	145—1
5' 5"	144—4	146—3	148—2	149—1
5' 6"	148—4	150—3	152—1	153—1
5' 7"	152—4	154—3	156—1	157—0
5' 8"	157—5	159—4	161—2	162—0
5' 9"	162—6	164—5	166—3	167—1
5' 10"	167—8	169—7	171—5	172—2
5' 11"	172—10	175—9	177—7	178—4
6' 0"	178—13	181—12	183—10	184—7
6' 1"	184—16	187—15	190—13	191—10

Female weight, subtract number of pounds designated.

## I. NORMAL BODY WEIGHT IN POUNDS AND KILOGRAMS

The normal body weight in pounds is obtained from an age-height-weight actuarial table (Table I) in which the figures represent weights for males, female weights being obtained by subtracting the small figures opposite each weight.

The conversion table (Table II) converts this figure from pounds to kilograms by dividing by 2.2.

TABLE II  
CONVERSION TABLE

BODY WEIGHT		BODY WEIGHT		BODY WEIGHT	
LB.	KILO.	LB.	KILO.	LB.	KILO.
15.4	7	83.6	38	151.8	69
17.6	8	85.8	39	154.0	70
19.8	9	88.0	40	156.2	71
22.0	10	90.2	41	158.4	72
24.2	11	92.4	42	160.6	73
26.4	12	94.6	43	162.8	74
28.6	13	96.8	44	165.0	75
30.8	14	99.0	45	167.2	76
33.0	15	101.2	46	169.4	77
35.2	16	103.4	47	171.6	78
37.4	17	105.6	48	173.8	79
39.6	18	107.8	49	176.0	80
41.8	19	110.0	50	178.2	81
44.0	20	112.2	51	180.4	82
46.2	21	114.4	52	182.6	83
48.4	22	116.6	53	184.8	84
50.6	23	118.8	54	187.0	85
52.8	24	121.0	55	189.2	86
55.0	25	123.2	56	191.4	87
57.2	26	125.4	57	193.6	88
59.4	27	127.6	58	195.8	89
61.6	28	129.8	59	198.0	90
63.8	29	132.0	60	200.2	91
66.0	30	134.2	61	202.4	92
68.2	31	136.4	62	204.6	93
70.4	32	138.6	63	206.8	94
72.6	33	140.8	64	209.0	95
74.8	34	143.0	65	211.2	96
77.0	35	145.2	66	213.4	97
79.2	36	147.4	67	215.6	98
81.4	37	149.6	68	217.8	99
				220.0	100

2.2 lbs. = 1.0 Kilo.

## II. CALORIC ALLOWANCE PER KILOGRAM

For an average male diabetic patient, 30 calories per kilogram normal body weight are allowed; for an average female diabetic patient, 25 calories per kilogram normal body weight. These figures are used for the average type of diabetic individual whose work is light in character, as is true of the business and professional worker and the housewife. Their life is spent mainly indoors, their greatest exertion that of walking about. More calories are unnecessary for such a man, and women usually require less calories than men.

Based on physical activity, the following allowances are used per kilogram normal body weight.

For a bed patient, 25 calories  
 For light work, 30 calories  
 For medium work, 35 calories  
 For heavy work, 40 calories

## III. TOTAL CALORIC ALLOWANCE

This is obtained very simply by multiplying I by II; i.e., kilograms normal body weight multiplied by caloric allowance per kilo yields total caloric allowance per day.

## IV. THE DIET FORMULA

This is the simple method used to convert the total caloric allowance into carbohydrate, protein, and fat.

*Protein.*—For a man, one gram per kilo normal weight is allowed. For a woman, either three-quarters or two-thirds of a gram per kilo is sufficient. More than a gram of protein per kilo is unnecessary and wasteful. Less than two-thirds gram per kilo may be detrimental in not supplying enough protein to replace normal breakdown of muscle structure. It is well known that we in the United States consume a large per capita proportion of meat, much more than is physiologic. For the diabetic person, re-education in the matter of meat eating is a necessity. And in these days of meat rationing, a little re-education in how much meat we really need per day would be serving two purposes, nutritional and patriotic.

*Fat.*—In order to keep the diet as close as possible to its 20 per cent of caloric content in fat, an arbitrary limit on the fat in the diet must be set. The average American likes his fat and hence eats too much of it, and it also is a rationed item today. Therefore, the fat in our diabetic diet has been fixed at a maximum of 90 grams per day. This figure allows enough butter, cream, and milk for the average individual, and more is hardly ever necessary. Figures as low as 70 grams may be used without resorting to artificial fatlike substances such as mineral oil for frying and salads. But diets using as little as 40 or 50 grams are unsatisfactory to the patient and are usually abandoned by him soon after leaving the doctor's immediate supervision in the hospital.

*Carbohydrate.*—Having determined the protein and fat in the diet, the remainder of the calories are simply prescribed as carbohydrate.

Such a calculation takes about a minute or two for any patient. The procedure is best illustrated by an example, taking the steps in their proper order.

Assuming we are dealing with a patient who is 42 years old, 67 inches tall, and is a salesman by occupation:

1. Normal body weight should be 154 pounds or 70 kilograms.
2. Caloric allowance is 30 calories per kilo.
3. Total daily caloric allowance is  $70 \times 30 = 2100$  calories.
4. Protein: 1 gram per kilo = 70 grams.

Fat: = 90 grams

Protein calories =  $70 \times 4 = 280$  calories

Fat calories =  $90 \times 9 = 810$  calories

280 calories (protein) + 810 calories (fat) = 1090 calories

Total daily caloric allowance = 2100 calories

Calories supplied by protein and fat = 1090 calories

Calories to be supplied as carbohydrate = 1010 calories

Carbohydrate (grams) = 1010 divided by 4 = 250 grams.

*Diet:* carbohydrate, 250; protein, 70; fat, 90.

## INSULIN

The patient is given his maintenance diet, as outlined, for a period of four or five days, without insulin if possible. This is done in an attempt to evaluate the degree of severity of the patient's diabetes. In this hospital, we have arbitrarily set up three grades of severity of diabetes to match the Army's classification of three degrees of severity for most of the chronic diseases. The means used to control the patient's diabetes serves as the index of severity, as follows:

Mild, a diabetic individual requiring no insulin.

Moderate, a diabetic individual requiring up to 40 units insulin daily.

Severe, a diabetic individual requiring more than 40 units insulin daily.

In our experience, about one-third of the cases fall into the mild group in civilian practice. Our Army experience indicates that about half are mild cases, and most of the other half fall into the moderate group. This difference between diabetics in civilian and Army practice may be due to the fact that diabetics in the Army are discovered very early and that these cases receive prompt and adequate treatment in a hospital. It may be that this tends to halt or delay the natural tendency of the diabetes to progress and become more severe. Many such cases who have received insulin at some other Army hospital have been found to require less and in many cases no insulin after treatment at our hospital. A more detailed study of these cases is being prepared for publication at some future date.

Early in his hospitalization, the patient is taught to test his urine for sugar four times daily, once before each meal and on retiring, and to keep a record of these tests. In addition, he collects and measures his twenty-four hour urine output daily, and a quantitative analysis of the sugar present is done. The volume of urine in cubic centimeters multiplied by the per cent of sugar present yields the amount of sugar in grams that the patient is excreting daily.

Insulin dosage is based on this figure of grams of glucose excreted daily. All patients requiring insulin are started on protamine zinc only, allowing as a preliminary amount one unit of insulin for every 2 grams of glucose excreted. However, it is good policy not to prescribe less than 10 units of insulin daily. In other words, a patient excreting less than 20 grams of glucose per day usually can be made sugar-free by readjusting the carbohydrate among his three meals. This is where the four daily urine tests are of inestimable value. Ordinarily the patient receives three meals that are equal in carbohydrate, protein, and fat content. Most patients adjust themselves to this regime without difficulty. However, for the patient who is showing considerable sugar persistently after any one of the three meals, a slight adjustment in his meals may be made. This consists in shifting ten to twenty grams of carbohydrate from that meal to another, to a time of day when he is sugar-free. An adjustment of this sort has saved many a patient either from taking any insulin whatsoever or from an additional dose of another kind of insulin.

In our experience, few patients who require insulin are ever completely controlled on protamine zinc alone. In many cases, an additional dose of regular or crystalline insulin is necessary. We have only two requirements to propose when additional insulin is to be given. One is that this additional insulin dose shall never be more than one-third of the total insulin dose per



day and the other is that the additional insulin be given at breakfast time with the protamine zinc insulin rather than at some other time of day. We have always given the two kinds of insulin in the same syringe. In our experience, the effect is the same as when the two kinds of insulin are given separately. The only precaution to be observed is that of withdrawing the clear insulin first, followed by the protamine zinc insulin, so as to avoid injecting protamine into the other insulin bottle.

For the patient taking protamine zinc insulin, one other precaution is routine in this hospital. All such patients receive a small meal at bedtime, containing milk, to avoid depression of the blood sugar during the night to the reaction level.

A few words about blood sugars: They are taken three times weekly, before breakfast, in all diabetic patients. This is an extremely worth-while precaution in a patient taking protamine zinc insulin. Such a case may suffer insulin reactions, if blood sugars are not done. Treating diabetes without doing blood sugars is like making a suit of clothes without a pattern or a model. Each case must be individualized and all factors must be analyzed to achieve the maximum results.

Herein are graphically presented three cases of early diabetes seen at our hospital recently. They illustrate what the proper diet does, with and without insulin.

**CASE 1 (Fig. 1).**—This was a case of a 21-year-old soldier whose diabetes was discovered while in India, about one month after symptoms began. Note that his glucose tolerance had a peak of over 400 mg. blood sugar. He was placed on a high-fat diet: carbohydrate, 60; protein, 60; fat, 150; calories, 1,730. On this diet he was hungry and continued to spill sugar. Before being transferred to this hospital, his diet was raised to carbohydrate, 70; protein, 70; fat, 165; calories, 2,045. A second glucose tolerance test was better but still diabetic. He was still hungry. During this period of hospitalization he was sugar-free or showed only a trace of sugar for 26 out of his total of 40 days in the hospital.

He was transferred to this hospital on Feb. 14, 1943, and placed on a diet of carbohydrate, 230; protein, 70; fat, 90; calories, 2,010. This was about the same in total calories as his last diet in India. He was never hungry on this diet. A repeat glucose tolerance test showed more improvement. He was sugar-free or showed only a trace for 27 out of 28 days in the hospital.

Thus, despite a complete reversal of his dietary figures, representing an increase of 160 grams of carbohydrate over his previous diet, his diabetes was controlled better than before. And this increase of carbohydrate did not make necessary the addition of insulin. Finally, we had a satisfied patient.

**CASE 2 (Fig. 2).**—This was a case of a 36-year-old colored soldier whose diabetes was discovered about 6 weeks after symptoms began at a station hospital in a southern camp. He was placed on a diet of carbohydrate, 124; protein, 77; fat, 132; calories, 1,992. In addition, he was given regular insulin, four times daily, for a total daily dose ranging between 75 and 100 units. He was sugar-free or had only a trace of sugar for only 3 out of 23 days' hospitalization.

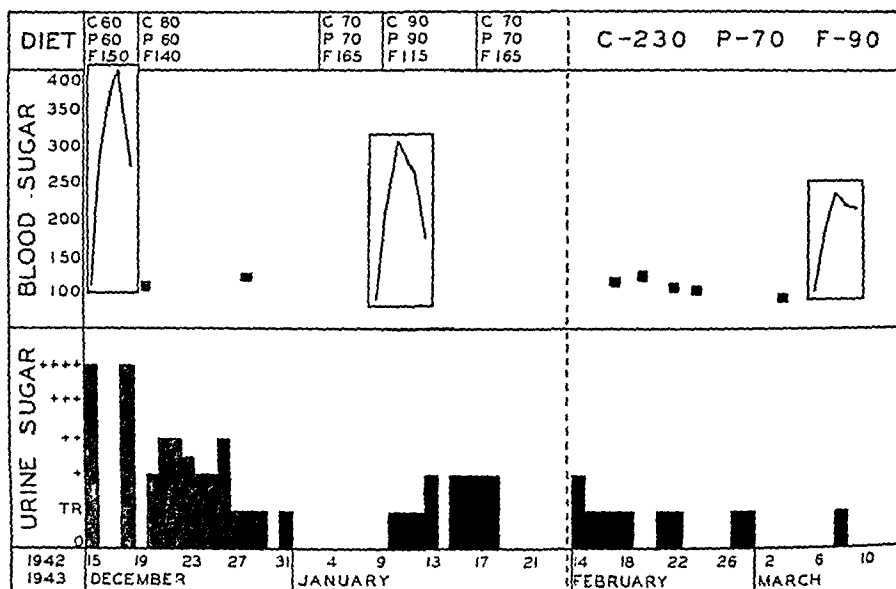


Fig. 1.

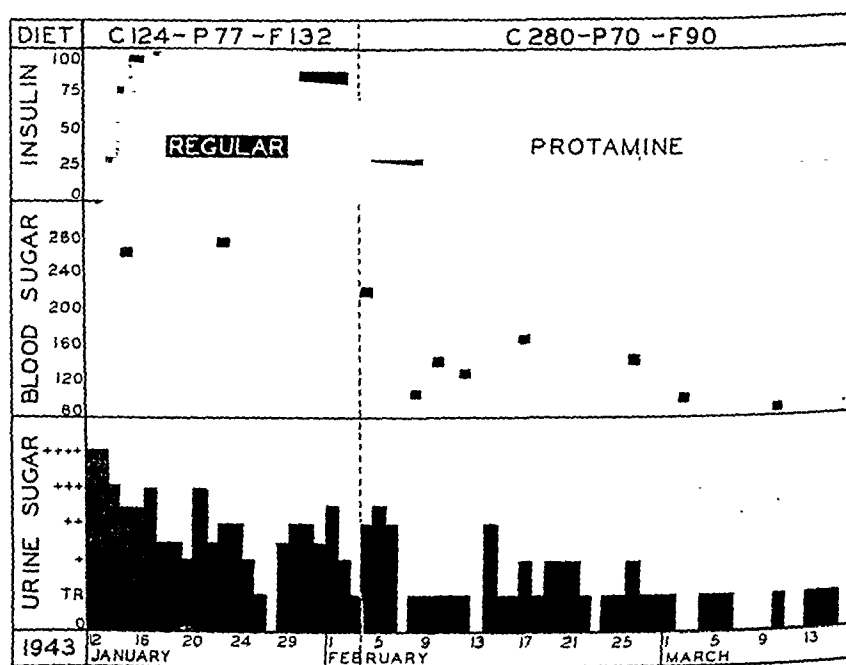


Fig. 2.

He was transferred to this hospital on Feb. 4, 1943, and placed on a diet of carbohydrate, 280; protein, 70; fat, 90; calories, 2,210. Since he was admitted on a dose of 75 units of insulin daily, it was felt wiser to give him 30 units of protamine zinc insulin, once daily, rather than omit insulin entirely. This dosage was reduced in five days to 25 units. His blood sugar promptly became normal. He was sugar-free or showed only a trace for 32 out of 41 days' hospitalization. An additional dose of regular insulin would have kept him completely sugar-free, but owing to the difficulties involved in teaching him insulin administration, it was felt wiser not to attempt to burden him with this extra task.

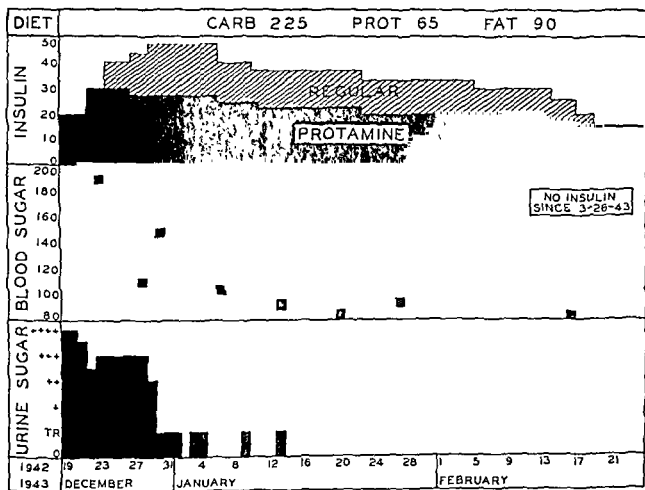


Fig. 3.

CASE 3 (Fig. 3).—This was a case of a 32-year-old soldier whose diabetes was discovered about 3 weeks after symptoms began, at this hospital. He was placed on a diet of carbohydrate, 225; protein, 65; fat, 90; calories, 1,970. At first he was given protamine zinc insulin alone, and after five days, regular insulin was added, to control after-breakfast glycosuria. After ten days of hospitalization, he was sugar-free with a normal blood sugar on 48 units of insulin daily. His insulin was then gradually reduced, and he was discharged on 20 units of protamine zinc insulin daily, still sugar-free. Subsequently he was followed for a time and his insulin progressively reduced, until he was on diet alone and sugar-free. His insulin was discontinued completely three months after his diabetes was first treated. This is not an uncommon occurrence in fresh cases of diabetes that are treated early with insulin. Whether it bespeaks a "cure" of such diabetes is still a problem for the future to decide.

However, it does indicate that those patients that require insulin should be given enough insulin to bring them quickly under control. Rather than increase the insulin dosage gradually to a maximum, we prefer to give the maximum insulin dose at the beginning of treatment and decrease it later, as necessary, thus perhaps discontinuing it entirely in the course of a few months.

#### SUMMARY

1. The diabetic diet should be a physiologic diet and based on known values of the normal diet.

2. The diabetic diet should be prescribed for the individual patient, according to his needs.

3. The diabetic diet is based on normal body weight, obtained from age-height-weight tables, and on the degree of physical activity of the patient.

4. The total caloric allowance is quickly and simply calculated for each individual patient.

5. By a simple method, the total caloric allowance per day is divided into carbohydrate, protein, and fat, in accord with physiologic values for a normal diet.

6. The principles and procedure for prescribing insulin are simplified.

7. Finally, three cases of early diabetes treated by these principles are graphically presented, illustrating the simplification of the treatment of diabetes.

## CIRCA 42, A NEW ITCH REMEDY\*

J. FRANKLIN YEAGER, PH.D., AND CHARLES S. WILSON, M.S., BELTSVILLE, MD.

THIS is a report of a new itch remedy, called Circa 42, designed to relieve the itching of skin when applied externally. It has been developed in response to an acute wartime need of a means of preventing or alleviating skin irritations caused by the attacks of insects such as chiggers, mosquitoes, etc., and of lessening thereby the danger of infection from scratching. At the authors' request, a number of persons suffering from chigger bites or itches that arose in other ways have tested Circa 42 on themselves. Written records indicate that Circa 42 has considerable promise as an efficacious itch remedy and should be brought to the attention of those interested in the treatment of pruritic skin.

The composition of Circa 42 is

n-butyl-p-aminobenzoate†	100 Gm.
benzyl alcohol	170 c.c.
anhydrous lanolin (melted)	20 c.c.
cornstarch	640 Gm.
sodium lauryl sulfonate	64 Gm.

plus additional benzyl alcohol as indicated below. The n-butyl-p-aminobenzoate is dissolved in the benzyl alcohol, which is first warmed, making an approximately saturated solution. The melted lanolin is added, and the mixture is kept warm and stirred until as much of the lanolin as will dissolve is in solution. While the liquid is warm, it is added slowly, a little at a time, to a thorough mixture of the cornstarch and the sodium lauryl sulfonate, and the whole is carefully kneaded to distribute the liquid evenly throughout the powder. An additional amount of benzyl alcohol, about a tenth of that already used, is added as before to give the material a desired consistency. The final preparation should be a doughy, nongreasy, cakelike material that can be packed in ointment jars or other suitable containers, although it is better not to use containers made of metal.

When the remedy is to be applied, the skin may be moistened slightly with water, depending on the physical consistency of the Circa 42. This material is rubbed onto the skin until it forms a moderately thick layer over the affected region. No attempt should be made to rub it vigorously into the skin. It will gradually dry to a powder, which should be left on the skin undisturbed. Effectiveness of treatment is lost when the material is removed from the skin by sweating, bathing, friction of clothes, etc.

The benzyl alcohol penetrates the skin quickly and produces a local anesthesia of short duration. The n-butyl-p-aminobenzoate penetrates more slowly, but has a more prolonged local anesthetic effect. When the alcohol of Circa 42

\*From the U. S. Department of Agriculture, Bureau of Entomology and Plant Quarantine, Agricultural Research Administration

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†Sold under the name "Butesin."

enters the treated skin, it takes dissolved benzoate in with it. The combined anesthetics produce a quick and prolonged local anesthesia. The lanolin helps to soften the skin and favors penetration, the sodium lauryl sulfonate facilitates spreading, and the cornstarch acts as an inert carrier base.

Records were obtained from 32 persons suffering from chigger bites and only 2 failed to obtain any relief. A total of 83 treatments were made. Of 76 records indicating quickness of relief, 61 showed that relief was obtained in less than 15 minutes, 13 in 15 to 30 minutes, and 2 in more than 30 minutes. Of 69 indicating duration of relief, 14 showed that relief persisted for less than 5 hours, 15 for 5 to 8 hours, and 40 for more than 8 hours; of the last, 13 showed that relief lasted from 8 to 48 hours, and 27, that the relief was permanent. In 18 instances permanent relief was obtained after only 1 application of the material; in 6 instances, after 2 applications; and in 2 instances, after more than 2 applications. Six of the 83 records did not indicate speed and duration, but all showed that some relief was obtained; 8 were interrupted by loss of the material from the skin because of bathing, sweating, and friction from clothes, but not before some relief from the itching was obtained.

The material was tested also on skin itching from other causes. Seven persons reported on 14 treatments made for relief of mosquito bites; 7 of these treatments were each followed by relief in less than 15 minutes, 6 in 15 to 30, and 1 in more than 30 minutes. Each of 9 treatments gave relief lasting less than 5 hours, and each of 4 gave permanent relief.

Permanent relief of discomfort from a yellow jacket sting was reported by one person, the relief occurring in less than 15 minutes. Another person treated "spider" bites. Each of 4 treatments gave relief in less than 15 minutes; 3 gave relief for 5 to 8 hours, and permanent relief from 1 bite was had after 2 treatments.

Five persons reported on use of the material to relieve itching from unrecognized causes. Of the 11 treatments, 9 gave relief in less than 15 minutes, 1 in over 30 minutes, and 1 gave no relief at all; 1 gave relief lasting less than 5 hours, and each of 8 treatments gave relief for over 8 hours. In two instances the relief lasted indefinitely, in one case after 1 treatment, in the other, after 2.

Two persons reported on treatment of poison ivy itch with Circa 42. Each of 2 treatments gave relief in less than 15 minutes, and each of 2 others in 15 to 30 minutes. Relief lasted less than 5 hours in 2 instances, 5 to 8 hours in 1, and indefinitely (after a single treatment) in 1 instance.

Similar results were indicated verbally by a number of other persons who used Circa 42 to relieve itching from chigger bites, mosquito bites, poison ivy, and unknown causes.

Equally promising results have been obtained by personnel of the United States Army,\* who have made preliminary tests wherein Circa 42 was used in the tropics to relieve itching caused by chigger bites and fungus infections.

*Conclusions.*—These tests indicate that Circa 42 shows considerable promise as a remedy for the relief of itching skin and is worthy of more extensive trials by those interested in the treatment of pruritus.

\*Permission to refer to those results has kindly been granted by the Office of the Surgeon General, Washington, D. C.

## CLINICAL USE OF ORAL THERMOMETERS\*

### REPORT OF STUDY TO DETERMINE TIME REQUIRED FOR RELIABLE REGISTRATION

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ALTHOUGH many scientific papers have been written on the subject of clinical thermometers, one question has remained unanswered: What is the basis for the time designations ("1 minute," " $\frac{1}{2}$  minute," "60 seconds," etc.) used on oral clinical thermometers? In an effort to gather information on this subject, a questionnaire was sent to 100 training schools for nurses throughout the country. This questionnaire asked: (1) "What is the minimum time interval between insertion and reading of clinical thermometers?" and (2) "Have the above time intervals been selected on the basis of the manufacturers' statements, your own clinical tests, common knowledge, or some other basis?" Of the 69 replies to the first question, 27 answered that the time of insertion of the thermometer in recording oral temperature was less than three minutes; 37 stated three minutes; and 5 recommended longer than three minutes. In reply to the second question, 13 stated that these time intervals were based partially or wholly on manufacturers' statements; 30 on clinical test; 26 on common knowledge; 16 on textbook statements, and 10 on experience. Several of the replies to the second question indicated that the basis of selection of a time interval had been obtained from more than one source. Since there seemed to be such wide variance among professional persons most familiar with the taking of oral temperatures, and since the time designations on the instruments have been interpreted by lay users to indicate the time necessary for registration of the actual temperature of the patient, it was decided to undertake a study to determine the time required for oral clinical thermometers to reach equilibrium† in the mouths of individuals under ordinary conditions of use.

Approximately 1,000 clinical thermometers were collected by the Food and Drug Administration throughout the United States in the Spring of 1940. Of these thermometers, about 800 were found to meet the requirements and tests specified for clinical thermometers in the Commercial Standard CS 1-32 of the United States Department of Commerce, Bureau of Standards. By random sampling of these 800 thermometers, 20 instruments were selected for testing.

Realizing at the outset that there might be wide variations in the time required for individual instruments to reach equilibrium, preliminary investigations were conducted to ascertain the effect of various media with different conductivity coefficients on the form of the "time-temperature curve" for oral

\*From the Food and Drug Administration, Federal Security Agency, Washington, D. C.

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†Equilibrium of an instrument was considered for our purposes to be that temperature reading within one-tenth of one degree of the registration of the instrument after five or six minutes of insertion in the media.

clinical thermometers. In these preliminary studies simultaneous observations of time and temperature readings on oral clinical thermometers were made under laboratory conditions in different media at various temperatures. Subsequently, the clinical experiment was undertaken. The details and results of both the laboratory and clinical experiments are given below:

#### WATER BATH EXPERIMENT

These 20 thermometers were placed, in pairs, in a water bath which was maintained at a constant temperature by means of a thermostat and a device to keep the water in circulation. A reading was taken of each instrument every thirty seconds for six minutes.\* The bath was maintained at 98° F. until all the thermometers were read, then at 100° F., 102° F., and finally 104° F. Conditions were carefully controlled so that as few extraneous factors as possible would influence the results. Some of these factors were: depth of immersion of the thermometer in the water, angle of vision while reading the instrument, interval of testing during the heating cycle of the bath, and the human factor (all readings by the same person) in reading the instrument.

It was noted that the mercury rose rapidly, and although each instrument reached equilibrium within ten seconds, the water bath experiment gave certain information concerning the variation of the final readings among thermometers. It appeared that the thermometers were slightly more variable at lower temperatures since the standard deviation at 98° F. was 0.13° and decreased as the temperature was increased, until at 104° F. it was 0.09°. (The ratio of these variances is just significant statistically and indicates a possible trend.)

As the rise of mercury in the thermometers was too rapid in this medium to obtain a satisfactory "time-temperature curve," it was decided to repeat the experiment in another medium with a lower conductivity coefficient.

#### AIR-BATH EXPERIMENT

A practical medium with a lower conductivity coefficient than water is air. Testing was carried out in a thermostatically controlled air bath, with the temperature maintained at 106° F. The 20 thermometers were inserted in the air bath singly. The time required for the mercury column to rise from 94° F. to 95, 96, 97, 98° F., etc., was recorded. It was observed that all the thermometers reached equilibrium in ninety seconds. Nevertheless, the thermometers were retained in the air bath for six minutes to make certain that the instruments had reached equilibrium. Each instrument was rechecked after an initial test. Such factors as depth of insertion of the instrument in the air bath, angle of vision while reading the instrument, interval of testing during the heating cycle of the bath, and the human factor in reading the thermometer were controlled.

The readings of the temperature and time in seconds are plotted for a single typical thermometer in Fig. 1. It is noted that about one and one-half minutes were required for an oral clinical thermometer to reach equilibrium in an air bath. The repeated observations on the same thermometer closely dupli-

\*All timing throughout the investigation was done with a stop watch calibrated in two-tenths of a second.



cate those of the initial readings. Considering the actual data and theoretical aspects, a smooth curve was fitted to the data. It is of the form

$$Y = T_f - (T_f - T_i) e^{-bx}$$

where  $T_i$  is the initial temperature reading of the thermometer (here  $94^\circ \text{F.}$ ),  $T_f$  is the final temperature, and  $Y$  is the temperature at any given time ( $x$ ).

In terms of logarithms, the equation means that a straight line with slope " $b$ " could be fitted to plotting of the logarithms of the relative change in temperature against time.

All 20 thermometers were tested in the same manner, and it was found that this type of equation was the best fitting in each instance.

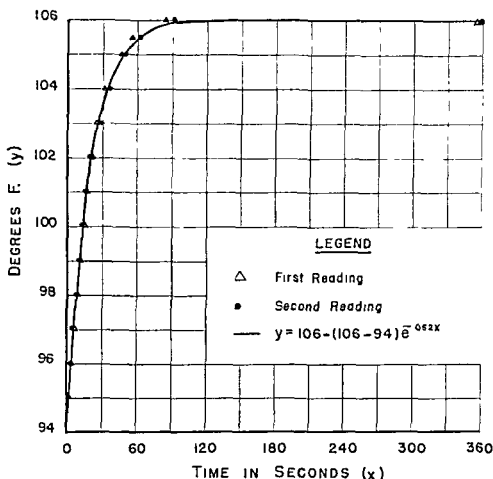


Fig. 1.—Curve fitted to thermometer readings in air bath constant at  $106^\circ \text{F.}$

#### CLINICAL TESTING

From these determinations in the air bath, the 20 thermometers under consideration were arbitrarily classed as "slow" and "fast." It was noted that some of these instruments bore a time designation and that about half were of the "regular" bulb type and the remainder "stubby" bulb instruments. Consequently the 8 thermometers picked from the group of 20 for use in our clinical study were separated into 8 groups according to these three physical characteristics. This choice and classification were made primarily to insure that the various types of oral thermometers were equally represented in the clinical test.

Since it was realized that physiologic variations in individual subjects might influence the recording time, a preliminary clinical investigation in which a thermometer was inserted in a patient's mouth for periods of from one second to ten minutes showed that it is possible to repeat temperature readings closely even when the thermometer is inserted for less than one minute. However, these readings were not the patient's "actual" temperature as indicated

by the reading of the instrument after five or six minutes. The point to which the mercury was shaken down seemed to make little difference as long as the meniscus was below 95° F. From this experience, the following "time intervals" were chosen for use in the clinical testing of the 8 thermometers: (1) five seconds; (2) ten seconds; (3) twenty seconds; (4) thirty seconds; (5) forty-five seconds; (6) sixty seconds; (7) ninety seconds; (8) one hundred and twenty seconds; (9) one hundred and eighty seconds; (10) two hundred and forty seconds; (11) three hundred seconds; (12) three hundred and sixty seconds.

It was noted during these preliminary clinical studies that certain activities appeared to affect the registration of the temperature. Taking hot drinks and smoking produced transitory elevation, and the taking of cold drinks caused a temporary lowering of local mouth temperature, which required from fifteen to thirty minutes to return to "normal." Consequently, suitable precautions were taken to control the subject's activities directly before and during the regular periods of testing.

In the clinical study of a group of 16 healthy girls, 8 white and 8 Negro, and a group of 16 healthy boys, 8 white and 8 Negro, all between the ages of 14 and 20 years, were used. Since the subjects were domiciled in training schools, their activities and diets were comparable throughout the periods of testing. These 32 subjects were taught the proper method of using an oral clinical thermometer. The 8 selected thermometers were tested and rechecked on all the subjects in both groups. The thermometers were read, temperatures recorded, and the instruments shaken down by us to a point near their original readings (below 95° F.) after each of the specified time intervals. One hour and fifteen minutes were required to complete an initial and recheck "set of readings" for each subject with any one thermometer. Each subject was tested about the same time of day in order to reduce the possibility of encountering the diurnal variation of the person's temperature.

The summary of the clinical tests consisted of 493 "sets of readings,"\* or 5,916 individual readings. (A "set of readings" is one subject's temperature record at all of the 12 time intervals as registered by any one instrument.)

The "time-temperature curve" for a single thermometer on a single subject was not as smooth as that for a single thermometer in the air bath. This is undoubtedly due to a slight physiologic variation in the subject and/or variations in the contact between the instrument and the surrounding tissue. However, an average "time-temperature curve" for several subjects on several thermometers is a smooth curve, although of slightly different type from that obtained when the thermometer is tested in an air bath.

Fig. 2 shows the average of the 493 readings plotted for each time interval, a smooth curve fitted to these averages, and, for comparison, a curve of the type applied to the data from the air bath experiment. The difference between the two curves can be seen readily. Although both curves seem almost identical for the first sixty seconds, the air bath curve continues in a horizontal line, while the curve for the averages of the clinical data continues to rise. This continued rise of the curve fitted to the clinical data may be due to a delayed rise of the mercury column in the thermometer caused by the slow return of

\*Because of circumstances beyond our control, only 493 complete "sets of readings" were obtained.

the tissues to body temperature after a possible slight cooling of these tissues on initial contact with the thermometer.

Having determined the "time-temperature curve" of a clinical thermometer under clinical conditions and having demonstrated the difference between the "time-temperature curves" for laboratory and clinical conditions, we concluded that we could proceed to determine the length of time necessary for a thermometer to approach within one-tenth of one degree of the "final" temperature under clinical conditions. "Final" temperature, for our purpose, is the average of the five- and six-minute readings of each set for each individual. Within each set of readings for 1 thermometer and 1 subject, differences were taken between each reading and its own "final" temperature. As will readily be seen, by using these differences the variation between thermometers and the variation between individuals are eliminated. This permits the tabulation of the difference between the actual reading at any given time interval and the reading finally attained.

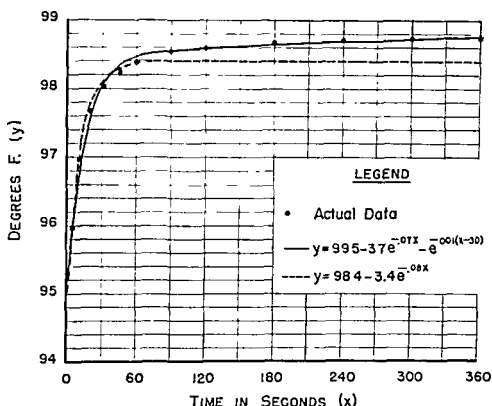


Fig. 2.—Curve fitted to averages of 493 "sets of clinical readings" as compared with a theoretical curve of the type derived from air bath data.  
unbroken line, clinical reading.  
broken line, theoretical curve derived from air bath data.

It can be seen from Table I that an oral clinical thermometer should remain in the mouth for three minutes (one hundred and eighty seconds), since the average of the 493 determinations required that amount of time to come within one-tenth of one degree of the final reading. In this study, in which 32 persons were used, the average three-minute temperature of the boys is 98.4° F. while that for the girls is 99° F.

A detailed statistical study of the differences from the "final" temperature by analysis of variance demonstrated that differences between sexes or between subjects were statistically significant in this clinical study. The differences between thermometers, however, were not significant even when they were separated into two groups; namely, those marked "1 minute" and those having no specified time designation.

The readings on the thermometers marked "1 minute" did not approach their own "final" readings significantly faster than the unmarked thermometers. At the end of three minutes, the thermometers with no time designations had an average difference of  $0.093^{\circ} \pm 0.010^{\circ}$  F. from the "final" temperature reading, while the corresponding figure for those with time designations was  $0.077^{\circ} \pm 0.011^{\circ}$  F. The type of bulb did not seem to cause any differences in the time required to reach the "final" reading. These figures for thermometers with no time designations on "regular" and "stubby" bulbs were, respectively,  $0.105^{\circ} \pm 0.013^{\circ}$  and  $0.081^{\circ} \pm 0.016^{\circ}$  F., while for thermometers with time designations, the averages for "regular" and "stubby" bulbs were, respectively,  $0.065^{\circ} \pm 0.015^{\circ}$  and  $0.088^{\circ} \pm 0.016^{\circ}$  F.

TABLE I

AVERAGE DIFFERENCES, STANDARD DEVIATION OF DIFFERENCES, AND GREATEST DIFFERENCE FROM "FINAL" TEMPERATURE FOR EACH TIME INTERVAL FOR THE 493 SETS OF READINGS

TIME INTERVAL SECONDS	DIFFERENCE FROM SUBJECT'S FINAL TEMPERATURE		
	AVERAGE DIFFERENCE, DEGREES F.	STANDARD DEVIATION DEGREES F.	GREATEST DIFFERENCE, DEGREES F.
5	2.792	1.146	6.8
10	1.780	0.899	4.8
20	1.083	0.648	3.8
30	0.731	0.485	3.6
45	0.511	0.398	3.2
60	0.367	0.332	2.6
90	0.225	0.251	1.5
120	0.151	0.251	1.6
180	0.083	0.169	0.9
240	0.044	0.159	0.8*

\*There was one very unusual difference greater than 0.8. This figure was 1.7.

#### APPLICATION OF RESULTS

On June 12, 1941, the Food and Drug Administration issued a trade notice to manufacturers and distributors of clinical thermometers in reply to numerous requests concerning the type of information that should appear on clinical thermometers and in the circulars accompanying the instruments. In order to obtain uniformity of information in the material to accompany the instruments which would not meet with adverse criticism under the provisions of the Federal Food, Drug, and Cosmetic Act, a circular entitled "Directions for Use of Thermometer" was provided as a guide. The text of this circular included information on how to read, shake down, use, and care for a thermometer and interpret temperature readings as well as information regarding the accuracy of the instrument. Copies of this circular may be obtained, upon request, from the Food and Drug Administration, Federal Security Agency, Washington, D. C.

#### CONCLUSIONS

1. The character of the rise of the mercury column in an oral thermometer under clinical conditions is different from that under laboratory conditions.
2. In this study, thermometers marked with specific time designations "1 minute," " $\frac{1}{2}$  minute," and "60 seconds" were found to require about the same length of time for the instrument to reach equilibrium as thermometers with no time designations.
3. Three minutes should be the minimum time interval allotted for an oral thermometer to reach equilibrium under ordinary conditions of use.

# ELLIPTOCYTOSIS, A REPORT OF TWO CASES\*

MARY K. HELZ, M.A., AND MAUD L. MENTEN, M.D., PH.D., PITTSBURGH, PA.

**E**LLIPTOCYTOSIS is a rare morphologic anomaly of human red blood cells. The condition was first described by Dresbach<sup>1</sup> in 1904 and apparently has no pathologic significance. Wyandt and her associates<sup>2</sup> in 1941 presented a total of 332 cases, composed of 86 persons studied by them and 246 individuals previously reported. In the following report, two additional individuals, an American Negro baby and his father with this anomaly, are added to the list. A detailed study of blood of the baby indicated a progressive increase in the degree of elliptocytosis during the first four months of life.

A comprehensive bibliographical survey of elliptocytosis has been given by Wyandt, et al. and need not be repeated here. No additional references have been found in the literature.

The pertinent findings of our cases are outlined below:

**CASE 1.**—The patient, a male aged two and one-half months, was admitted to the Children's Hospital of Pittsburgh on August 30, 1942, with a history of diarrhea, malnutrition, and a fever of two and one-half days' duration. He was weak and dehydrated. During his seven weeks' stay in the hospital he gained weight slowly and, except for the period between October 10 and October 17, 1942, when he developed an upper respiratory infection and a bilateral otitis media, he maintained a subnormal temperature. Bacteriologic findings and urinalysis were irrelevant. At the time of his discharge on October 23, 1942, he appeared much improved. During the first routine blood examination a considerable number of oval red cells were discernible in the counting chamber and a tentative diagnosis of elliptocytosis was made. Blood examination during his period of hospitalization, together with two blood counts at a later date, is summarized in Table I. The leucocytosis occurring during the early hospitalization was probably related to otitis media. The cause of the lymphocytosis on the last examination was not ascertained.

TABLE I

DATE	Hb. IN GRAMS	R B C IN MILLIONS	W B C IN THOUSANDS	NEUTROPHILES %	LYMPHOCYTES %	MONOCYTES %	MYELOCYTES %	METAMYELOCYTES %	EOSINOPHILES %
8/31/42	10.0	3.4	14.2	41	40	1	6	10	2
9/11/42	8.8	3.5	12.9	49	48	2	1		
9/30/42	9.2	3.75	11.7	69	25	1	1		4
10/14/42	7.5	3.1							
10/23/42	8.2	2.95	6.8						
12/1/42	8.1	2.9	9.1	54	40	1		4	1
4/1/43	10.0	3.61	14.1	27	69		1	2	1

On December 1, 1942, the patient, apparently well, was readmitted for further blood study. Blood platelets averaged 486,000 per c. mm. The reticulocytes showed a value of 1.8 per cent, and none of these appeared oval. Fragility tests gave a range of hemolysis between 0.50 per cent and 0.30 per cent sodium chloride. Increased oxygen and carbon dioxide tension and washing with lecithin produced no appreciable change in the appearance of the

\*From the Departments of Pathology, University of Pittsburgh, and Children's Hospital. Received for publication, July 30, 1943.

erythrocytes. Enumerated erythrocytes in stained smears were divided into three groups on the basis of the following measurements: namely, round cells ( $6$  to  $8.5\ \mu$ ), oval cells ( $9 \times 6\ \mu$  to  $6.5 \times 5\ \mu$ ), and sausage-shaped cells ( $11 \times 3.2\ \mu$  to  $7 \times 5\ \mu$ ). Comparison of the percentile distribution of the cells in the three groups is shown in Table II.

TABLE II

DATE	HB. IN GRAMS	R B C IN MILLIONS	NO. CELLS COUNTED	% ROUND	% OVAL	% SAUSAGE
9/11/42	8.8	3.5	400	75	25	
9/30/42	9.2	3.75	400	67.5	32	0.5
10/14/42	7.5	3.1	400	76	22.5	1.5
10/23/42	8.2	3.75	400	33	59	8
12/ 1/42	8.1	2.9	2000	36	51.9	12.1
4/ 1/43			2000	34.6	52.6	12.8

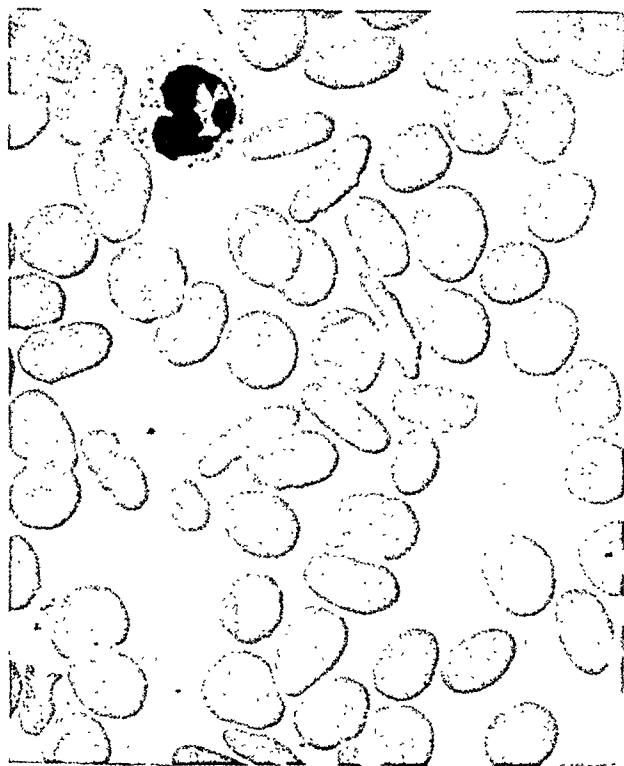


Fig. 1.—Photomicrograph of blood smear showing elliptical cells. (Wright and dilute Giemsa stain.)  $\times 1200$ .

It is concluded that stability in the development of elliptocytosis was reached about the fourth month, because a progressive increase in the percentage of sausage-shaped cells was observed up to but not beyond this time (Fig. 1). After the fourth month, constant percentages of oval and sausage forms were obtained. The interruption in the progressive increase in degree of elliptocytosis shown on October 14, 1942, is probably due to infection. Sternal marrow showed all nucleated red cells to be round.

CASE 2.—The father of this patient, aged 25 years, was the only member of this family available for study who showed elliptocytosis. One complete blood count established the diagnosis, but no opportunity was presented to study his blood in detail. His stained smears showed no sausage-shaped forms, but a fair percentage of oval forms. Complete blood

counts made on the mother, a paternal uncle and his baby, and the paternal grandmother showed nothing of interest.

#### DISCUSSION

The most interesting feature was the progressive increase in the degree of elliptocytosis up to the fourth month, when the condition apparently became stabilized. Wyandt et al.<sup>2</sup> and Hunter<sup>3</sup> reported that the anomaly was present at birth with an increasing degree of elliptocytosis as growth of the child proceeded. Neither investigator stated when the degree of development was complete. Hunter was unable to follow the increased abnormality of the shape of cells beyond the third month because of the sudden death of the child.

A possible explanation for the decreased percentage of elliptical cells during infection, as shown by the count on October 14, 1942, may be the fact noted in transfusion experiments by Vischer<sup>4</sup> that these abnormal cells have a shorter life span of twelve to thirteen days, as compared with the approximate thirty days' life span of the normal red cells, and hence may be more readily removed from the circulation. This more rapid destruction would result in a greater number of young normal erythrocytes in the peripheral circulation and thus a relatively lower percentage of oval forms.

The nucleated red blood cells in the sternal marrow and reticulocytes in the circulating blood did not exhibit elliptical contour. This observation agrees with the findings of Cheney,<sup>5</sup> Leitner,<sup>6</sup> and Terry et al.<sup>7</sup> in contrast to those of Florman and Wintrobe<sup>8</sup> who noted 0.2 per cent elliptical reticulocytes in bone marrow. Altering environmental conditions, as with increased O<sub>2</sub> and CO<sub>2</sub>, produced no alteration in the elliptocytes.

#### SUMMARY

Two cases of elliptocytosis are reported. In one of these, a progressive increase in the degree of elliptocytosis up to the fourth month was noted.

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10/23/42	8.2	3.75	400	33	59	8
12/ 1/42	8.1	2.9	2000	36	51.9	12.1
4/ 1/43			2000	34.6	52.6	12.8

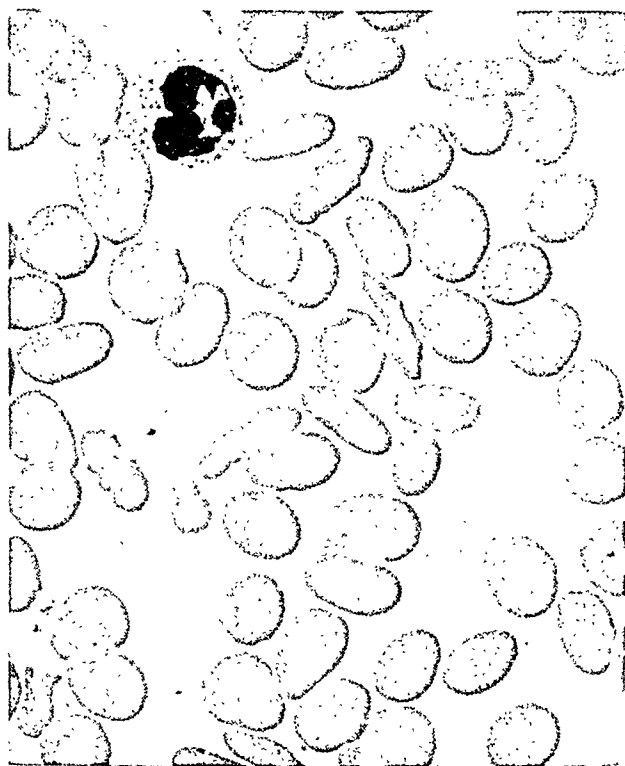


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CASE 2.—The father of this patient, aged 25 years, was the only member of this family available for study who showed elliptocytosis. One complete blood count established the diagnosis, but no opportunity was presented to study his blood in detail. His stained smears showed no sausage-shaped forms, but a fair percentage of oval forms. Complete blood



veloped. The following morning, the patient was stuporous, and by afternoon was comatose. Marked bleeding now occurred after cleaning his teeth or after any minimal trauma to the mouth.

By the use of transfusions from donors who had received vitamin K, whole blood transfusions, plasma, vitamin K, and other measures, the bleeding was controlled and the patient improved. The prothrombin increased to near normal, the icteric index gradually diminished to 220 by July 15, and the patient appeared improved. On July 21, it was decided to attempt a hippuric acid excretion test in an effort to determine the functional capacity of the liver. The routine test, as devised by Quick,<sup>2</sup> was used, the patient being given 6 Gm. of sodium benzoate in 30 c.c. of water, by mouth. The test was uneventful until four hours after the administration of the sodium benzoate, when the patient suddenly complained of severe pain across his upper chest, "as if somebody was stabbing me with a knife," extreme dyspnea, and orthopnea. His blood pressure earlier in the morning was 106/70; but now it was 150/120 and the pulse was rapid and thready. The patient rapidly went into shock, with the pulse becoming imperceptible, and lapsed into a semicomatose state; then by the use of plasma, oxygen, and stimulants, he recovered from the shock and felt better within a few hours. Electrocardiogram, x-ray of the chest, physical examination, urine, chemical studies of the blood, and other examinations were all essentially as before the test, except that the icteric index had gone up to 275. The following day, on July 22, the patient's condition was further improved, and repeated studies failed to reveal a cause of the episode.

A rather rapid survey of the literature revealed no report of untoward systemic reactions from sodium benzoate, and it was thought that the episode was incidental and not due to the drug. The first test was not completed, and as the condition of the patient was satisfactory, the test was repeated on July 23, the patient again being given 6 Gm. of sodium benzoate in 30 c.c. of water, by mouth. At approximately the same time, four hours after ingesting the chemical, the patient again complained of severe chest pain, this time across the lower chest. Extreme dyspnea and orthopnea occurred a few minutes later, with the blood pressure rising to 155/126. The patient again rapidly went into shock, requiring intravenous glucose, plasma, oxygen, and stimulants for relief. During the shock his condition resembled that of a patient with a rather large pulmonary embolus. He could not be aroused for about twenty minutes and was stuporous and confused for four hours. Again the patient made a recovery from the shock, but this time only partially regained his former condition, feeling much weaker, appearing rather ill, and lacking his usual appetite and good spirits. Again, a thorough study was essentially as before the reaction, except for the icteric index, which had increased from 220 on July 15 to 275 on July 22, and to 285 on July 24. This was interpreted as an indication of increased damage to the liver, as there was no evidence of hemolysis of a large number of red blood cells. After the second episode, it was felt that both reactions were due to the sodium benzoate. This was further substantiated by the fact that the patient had received only vitamin K, vitamin B complex, choline chloride, transfusions, and plasma during his hospital stay, and continued to receive these during his recovery from each episode. The two reactions were essentially similar and occurred at almost exactly the same time after ingesting the drug.

After the first reaction, the white count was 4,450, with 40 per cent polymorphonuclears, 8 per cent eosinophiles, 7 per cent basophiles, and 46 per cent lymphocytes.

On July 26 he developed a cough, fever, sore throat, and malaise. Examination revealed mild redness of the throat and enlarged cervical glands. Otherwise, nothing was made out on physical examination aside from jaundice, small liver, and emaciation. The white blood cell count was 1,600, with 4 per cent eosinophiles, 1 per cent basophiles, 61 per cent lymphocytes, and 34 per cent monocytes. Repeated examinations of several smears showed no neutrophilic granulocytes. Tables I and II tabulate the white and differential blood counts, hemoglobin, red blood cell counts, liver, and transfusions during this period. One hundred units of liver extract and a 250 c.c. transfusion of whole citrated blood were given. On the following day, July 27, there were 2,400 white cells, again without any neutrophils, the differential showing 5 per cent eosinophiles, 2 per cent basophiles, 53 per cent lymphocytes, and 35 per cent monocytes. Several checkup counts, including the various vital

TABLE I  
BLOOD STUDIES

	DATE	HGB.	R.E.C.	W.B.C.	DIFFERENTIAL							LYMPHS.	MONOS.
					MYELOB.	MYELOS.	JUV.	STABS.	SEGS.	EOS.	BAS.		
1	6/2/42	92%		5,580	0	0	0	0	60	3	0	32	5
2	7/23/42	85%		4,450	0	0	0	0	40	8	7	45	0
3	7/26/42*			1,600	0	0	0	0	0	4	1	61	34
4	7/27/42*			2,400	0	0	0	0	0	5	2	58	35
5	7/28/42 A.M.	80%	4.08	2,650	0	0	0	0	0	4	1	60	35
6	(A.T.) P.M.	100%	4.58	3,200	0	0	0	0	2	9	0	39	50
7	7/29/42 A.M.	104%	5.50	4,700	0	0	2	2	1	5	2	74	15
8	P.M.			5,000	0	0	0	0	0	6	0	51	43
9	7/30/42 P.M.*	95%	4.14	5,000	6	30	0	0	9	7	2	31	14
10	7/31/42 A.M.	95%	4.72	7,050	1	30	4	4	13	8	2	24	15
11	P.M.	100%	4.71	7,350	1	12	1	14	11	7	4	41	9
12	8/1/42 A.M.	100%	5.33	7,500	0	10	3	6	30	17	2	28	4
13	(A.T.) P.M. *		5.32	8,300	0	2	0	12	30	9	1	33	13
14	8/2/42	95%	5.20	8,150	0	2	5	11	23	6	0	40	13
15	8/3/42 A.M.	95%	5.50	6,500	0	0	3	8	38	6	1	31	13
16	P.M.			6,150	0	2	4	7	35	5	1	34	12
17	8/4/42 A.M.			8,350	0	0	0	0	62	1	2	35	0
18	P.M.			11,100	0	2	3	8	37	6	2	29	13
19	8/5/42	85%		14,700	0	4	6	11	49	2	1	24	3
20	8/6/42	90%	5.15	10,200	0	3	1	2	54	5	2	28	5
21	8/10/42 A.M.			20,350	0	0	0	0	91	0	1	9	0
22	P.M.	95%		36,600	0	0	2	27	66	0	0	5	0
23	8/11/42			18,950	0	0	0	25	70	0	0	5	0
24	8/12/42			14,100	0	0	0	10	62	11	2	9	6
25	8/13/42			10,050	0	0	0	7	61	5	2	18	7
26	8/14/42			9,950	0	0	1	1	62	8	3	22	3
27	8/15/42			13,350	0	1	1						

\*Mean average of 9 complete counts.  
A.T.—After Transfusion.

stains, continued to show complete absence of the neutrophilic series. The patient continued to receive whole blood transfusions, 200 units of intramuscular liver daily, and large amounts of all the vitamins.

On July 28, the white count was 2650 and was still without any of the neutrophilic series. The patient was much worse, the cough and fever had increased in severity, and the throat was now quite red and swollen, but without ulcers. The cough was productive of a greenish-yellow, foul sputum, with innumerable Vincent's organisms, but with no pus cells.

By July 29, the patient had not improved, and a sternal biopsy by the punch method was made. This revealed an arrested maturation of the myeloid elements of the bone marrow, except eosinophiles and basophiles. Peculiarly enough, these two constituents were found in normal or increased numbers, mostly in mature form. The amount of intramuscular liver injected was increased, and the small blood transfusions were given more frequently. The blood count gradually increased after this so that forty hours after the increase in liver dosage, the total white count was 5000, with 6 per cent myeloblasts, 30 per cent myelocytes, 9 per cent segmented neutrophilic forms, 7 per cent eosinophiles, 2 per cent basophiles, 32 per cent lymphocytes, and 11 per cent monocytes.

The bone marrow apparently regained its normal ability to mature granulocytic neutrophils, since, when the patient developed a severe acute systemic infection on Aug. 10, the white blood count rapidly rose to 36,600, with 95 per cent granulocytes. The infection was controlled by the use of sulfadiazine, without effect on the bone marrow, but he eventually died on Sept. 11, 1942, from liver failure. The icteric index gradually decreased from 285 to 200, and remained at approximately this level until death. Autopsy revealed severe yellow atrophy of the liver, marked degeneration of the corpus striatum of the brain, marked wasting of the striated muscles, and a normal bone marrow.

#### DISCUSSION

There are many reports of the use of sodium benzoate in the treatment of the rheumatic state, and more recently for the determination of hepatic function in the hippuric acid excretion test. Bryan<sup>1</sup> in 1925 stated "A review of the literature indicated that normal human beings may completely convert at least 25 grams of sodium benzoate to hippuric acid without toxic effect and with the appearance of only traces of free benzoic acid in the urine." Quick<sup>2</sup> felt that sodium benzoate was a safe drug in moderate amounts when he devised the hippuric acid excretion test in 1933. Snell and Magath<sup>3</sup> feel that the hippuric acid excretion test is a test of the detoxifying function of the liver and is based on the conjugation by the liver of amino-acetic acid and benzoic acid into hippuric acid, but mention no harmful effect from its use. Bartels<sup>4</sup> repeated the test three times on most of 148 cases with thyroid disease, without untoward effect. Hirschheimer<sup>5</sup> did 246 tests on pregnant women, some complicated and others uncomplicated, without ill effect. The *Journal of the American Medical Association*<sup>6</sup> stated, "There is no direct or indirect evidence to indicate that sodium benzoate has any toxic effect on the liver. The drug was formerly used for the treatment of rheumatic disease and has been given to human beings in doses as large as 25 to 60 grams a day without producing any harmful effect. Quick's hippuric acid test has been performed on many patients with extreme degrees of injury to the liver without any appreciable toxic effects or without depressing other hepatic functions; slight gastric irritation has been noted in a few cases, but these symptoms are of a temporary nature." Quastel and Wales<sup>7</sup> found that there was low excretion of hippuric acid after both oral and intravenous administration of sodium benzoate in schizophrenia, but mentioned no ill effects. Davies, Wales, and Hughes<sup>8</sup> were of the same opinion and mentioned seventy-five cases

without complications. Londe and Probst<sup>9</sup> performed a modified hippuric acid test in many children without ill effect. Finkelman, Hora, Sherman, and Horwitt,<sup>10</sup> Campbell,<sup>11</sup> Vaccarro,<sup>12</sup> Snell and Plunkett,<sup>13</sup> Lipshutz,<sup>14</sup> Quick,<sup>15</sup> Haines, Magath, and Power,<sup>16</sup> and many others reported using this test in numerous cases, and none mentioned systemic reactions.

The absence of neutrophiles in the peripheral blood in this case, known by various names such as granulopenia, granulocytopenia, agranulocytosis, agranulocytic angina, leucopenia, or neutropenia, and which was described originally by Schultz<sup>17</sup> in Germany in 1922, and then by Lovett<sup>18</sup> in America in 1924, has not been described following ingestion of sodium benzoate. During the past twenty-one years, there have been numerous case reports and discussions, which have shown that with the increase in use of amidopyrine, arsphenamine, sulfonamides, and other substances depressing to the bone marrow, the incidence of granulocytopenia has increased. While a great many of these cases occur spontaneously, it is interesting to note that in most case histories there has been a preceding treatment of some type, by either a dentist or a physician. In Kracke's<sup>19</sup> series of 473 patients, nearly  $\frac{3}{4}$  had been receiving treatment for some other disease for a variable period prior to the attack of granulocytopenia.

Granulocytopenia in the various races has been discussed, and it has been found to be rare in the colored race, occurring eight times in the 473 cases collected by Kracke.<sup>19</sup> A review of the literature further reveals that it occurs in a ratio of about two female patients to one male.

The cause of granulocytopenia has been discussed at great length. The first investigators directed their efforts on the hypothesis that the cause was some type of infection, because various organisms were usually cultured from the lesions in the throat and blood stream. Also, Fried and Dameshek<sup>20</sup> were able to produce a type of primary granulocytopenia in rabbits by the injection of *Salmonella suispestifer*. Kracke<sup>19</sup> doubts that Fried and Dameshek reproduced a true granulopenic picture, but produced the picture which is commonly seen following injections of any killed organism. The more recent work is directed toward the fact that granulocytopenia is a disease of the bone marrow, and that the basic picture is the disappearance of the neutrophiles from the peripheral blood, which, in turn, is followed by the invasion of any and every organ that is accessible by organisms. According to Jackson (Cecil's Text),<sup>21</sup> the eosinophiles disappear early and reappear late. The total number of lymphocytes usually is reduced. Pus does not form during this stage. Reznikoff<sup>22</sup> feels that the following four factors are important in causing this disease: fatigue, drugs, menstruation, and infection. In the thirteen cases studied by him, he found that fatigue due to excessive work, lack of sleep, and worry was more frequently encountered than any of the other etiologic conditions, and it was also his opinion that monocytosis is an evidence of good prognosis when it occurs early and is persistent.

While the majority of cases are due to toxic action of a drug on the bone marrow, a smaller group is due to the toxic effect of an infection on the bone marrow, as for instance, typhoid fever, influenza, and measles, which are associated with a leucopenia. There are probably three causes: (1) a primary form with fever, ulcerative mouth lesions, and agranulocytosis, (2) malignant granulocytopenia of bacterial origin, and (3) malignant granulocytopenia of toxic origin. Ralph U. Leser,<sup>23</sup> in discussing agranulocytic angina due to the sulfon-

amide drugs, suggests treatment by parenteral liver therapy and by mouth, if possible; repeated daily transfusions of 200 to 300 c.c.; pentnucleotides; a high caloric diet; good nursing; adequate cardiac and respiratory stimulation; withdrawal of all analgesic drugs containing the benzene ring, and also all the so-called barbiturates. He feels that adenine sulfate is quite valuable and that local treatment of the oral lesions is important. Ottenberg<sup>24</sup> used transfusions from donors who had received typhoid vaccine to stimulate leucocytosis in treating leucopenia. He reports only one case and believes that the cure was unquestionably due to the treatment. He feels that this is the result not of the actual number of leucocytes transfused, but to the passive transmission of the bone marrow-stimulating factor. Dameshek and Wolfson<sup>25</sup> treated two cases of severe agranulocytosis with sulfathiazole in addition to transfusions, pentose nucleotides, and liver extract, feeling that the recovery, at least in part, was due to the effect of the sulfonamide drug on the sepsis, which was almost certainly present.

TABLE II  
TOTAL NUMBER OF WHITE CELLS OF EACH CLASS, AND TREATMENT

	W.B.C.	NEUTROPHILES	EOSINOPHILES	BASOPHILES	LYMPHS.	MONOS.	TREATMENT	
							TRANSFUSIONS	LIVER IN UNITS
1	5,850	3,510	176	0	1,872	292		
2	4,450	1,780	356	312	2,002	0		
3	1,600	0	64	16	976	544	250 c.c.	100 units intramus.
4	2,400	0	120	48	1,392	840	250 c.c. A.M. & 250 c.c. P.M.	200 units intramus.
5	2,650	0	106	26	1,590	928	250 c.c.	100 units intramus.
6	3,200	64	288	0	1,248	1,600	250 c.c.	100 units intramus.
7	4,700	188	235	94	3,478	705	250 c.c.	200 units intramus.
8	5,000	0	300	0	2,550	2,150	250 c.c.	200 units intramus.
9	5,000	2,250	350	100	1,600	700	250 c.c. A.M. & 250 c.c. P.M.	400 units intramus.
10	7,050	3,666	564	141	1,622	1,057	250 c.c.	200 units intramus.
11	7,350	2,866	515	294	3,014	661	250 c.c.	200 units intramus.
12	7,500	3,675	1,275	150	2,100	300	250 c.c.	200 units intramus.
13	8,300	3,652	747	83	2,739	1,079	250 c.c.	200 units intramus.
14	8,150	3,341	489	0	3,260	1,060	500 c.c.	400 units intramus.
15	6,500	3,185	390	65	2,015	845	250 c.c.	200 units intramus.
16	6,150	2,952	307	62	2,091	738	250 c.c.	100 units intramus.
17	8,350	5,146	83	166	2,905	0		100 units intramus.
18	11,100	3,550	666	222	3,219	1,443		100 units intramus.
19	14,700	10,290	295	147	3,528	441		100 units intramus.
20	10,200	6,120	510	204	2,856	510	500 c.c.	100 units intramus.
21	20,350	18,315	0	203	1,832	0	500 c.c.	
22	36,600	34,770	0	0	1,830	0	500 c.c.	
23	18,950	18,005	0	0	945	0	500 c.c.	
24	14,100						500 c.c.	
25	10,050	7,200	1,100	200	900	600		
26	9,950	6,732	495	198	1,832	693		
27	13,350	8,512	1,064	266	3,109	399		

Apparently the bone marrow varies in its appearance during the various stages of the disease, and there are at least two types of bone marrow seen in the early stages, one in which there is myeloid hypoplasia, and another in which there is arrest in maturation of the myeloid elements. From the literature one would assume that where myeloid hypoplasia exists, the prognosis is poor, whereas in those in which there is arrest of maturation of the myeloid elements, the outlook is better. Boyd<sup>26</sup> states "The bone-marrow varies in the fatal cases. As a rule it is aplastic and appears to be completely exhausted, as

is seen in experimental benzol poisoning. In others it is normal, and in still others it may be hyperplastic. In these latter cases there would appear to be a blockade of the marrow or a lack of maturation of the myelocytes, thus preventing the mature leucocytes from entering the circulation, a state of affairs comparable with what is found in pernicious anemia."

Kracke and Parker<sup>27</sup> in 1938 thoroughly reviewed the literature of drug therapy in relation to the neutropenic states, and cited eleven cases of granulocytopenia, nine with fatal termination resulting from the use of sulfanilamide. In all instances a large amount of the drug was administered over a relatively long period before the condition made its appearance. Rinkoff and Spring<sup>28</sup> in 1941 reported six cases illustrating the toxic effect of sulfonamides on the myeloid elements, three due to sulfanilamide, two to sulfapyridine, and one, a combination of sulfapyridine and neoprontosil. Three of their patients developed granulocytopenia within the first twenty-four hours or less of therapy, and three, after fourteen days or more of therapy. Three of these cases developing within twenty-four hours would mitigate against Kracke's experience that prolonged therapy with sulfanilamide was necessary to induce granulocytopenia.

The reactions occurring in our patient were probably of the so-called "anaphylactoid type." This term is used here to indicate a reaction closely resembling anaphylactic shock, but occurring after a chemical. The predominating symptoms of severe chest pain, extreme dyspnea, and orthopnea, early increase in blood pressure with later fall, rapid thready pulse, and shock, indicate the more common cardiac rather than pulmonary type of reaction. There apparently was no desensitization after the first reaction, as the second was as severe as the first. The increase in icteric index could be interpreted as the result of the anaphylactoid reaction, or as the direct result of chemical poisoning.

#### SUMMARY

This case presented various interesting problems. The patient apparently had a reaction following his first dose of sodium benzoate with some increase in his icteric index, probably due to further damage of the liver, and a second dose forty-eight hours later again caused a severe reaction and probably further increased the liver damage. The combination of the two doses produced an arrest of the maturation of the neutrophilic granulocytes in the bone marrow, but allowed the basophiles and eosinophiles to mature. Another patient with acute hepatitis and an even higher icteric index had no reaction from either the first or second test, performed at the same time as the case being reported here.

Liver damage, or increase in damage to an already diseased liver, following sodium benzoate, must be extremely rare, as we have been unable to find any report of cases in the literature. The increase in jaundice and the increase in general debility of the patient could be interpreted as indicating further damage to the liver either as a direct result of the chemical or as a result of the reaction.

As to the bone marrow and peripheral blood, the situation was also peculiar in that it is customary for the eosinophiles and basophiles to disappear from the peripheral blood, and their maturation to be arrested along with the

other granulocytes, but in this case they appeared in normal to increased numbers in the peripheral blood, and apparently matured normally in the bone marrow. It is impossible to say what part, if any, the severely damaged liver played in the granulocytopenia, except that there was response to very large doses of liver. A point against the liver having any particular part is the fact that later there was a white count of 36,600, secondary to a severe septicemia, showing that the bone marrow could respond even though a badly damaged liver condition was still present. Further proof that the liver probably played little part was the fact that the bone marrow was normal at autopsy even though very few normal liver cells remained.

#### CONCLUSION

1. A colored male with marked yellow atrophy of the liver had a severe reaction on two occasions, approximately four hours after ingesting sodium benzoate, manifested by excruciating substernal pain, elevation of blood pressure, dyspnea, and orthopnea, followed by shock.
2. Three days after ingesting the second dose of sodium benzoate, the patient developed signs of systemic infection and the white blood count showed an absence of the neutrophilic granulocytes, but a normal to increased number of eosinophiles and basophiles.
3. The bone marrow showed arrest of maturation of the neutrophilic myeloid elements, but normal maturation of the eosinophiles and basophiles.
4. There was later a marked leucocytic response to a generalized septicemia, showing complete recovery of the bone marrow.
5. The icteric index increased from 220 to 275 after the first reaction, and to 285 after the second reaction. The cause of the increase in icteric index after the reactions remains undetermined.

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# LABORATORY METHODS

## GENERAL

### THE EFFECT IN VITRO OF PROPYLENE GLYCOL ON ERYTHROCYTES\*

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THE fading from the microscopic field of erythrocytes suspended in equal parts propylene glycol and water led to the use of this diluent for the enumeration of white blood cells.<sup>1, 2</sup> The red cells remained clearly visible for about three minutes in the white cell counting chamber after whole blood had been diluted with propylene glycol and distilled water. In five minutes the red cells had almost completely disappeared, remaining as vague "ghost" forms at room temperature (20° C.) for as long as ten days.

Equally striking macroscopic changes were observed in the test tube when blood was mixed with equal parts propylene glycol and water. In three to five minutes after mixing, the initially turbid solution became clear, suggesting complete hemolysis. Earlier investigators have reported the hemolytic action of glycols, but this explanation of the disappearance of erythrocytes is refuted in the present report by dark-field microscopic examinations which showed the red cells still present in normal numbers in the apparently hemolyzed preparation. In higher concentrations of propylene glycol or in the presence of temperatures over 20° C., the faded red blood cells spontaneously reappeared. These observations led to the present study of the mode of action of propylene glycol, as hemolysis could no longer be considered the mechanism responsible for the initial fading of the red cells.

Bachem<sup>3</sup> in 1911 observed hemoglobinuria in animals following the intravenous injection of ethylene glycol, while the initial observation regarding the action in vitro of glycols on blood was made by Von Oettingen and Jirouch in 1931.<sup>4</sup> The hemolytic action of propylene glycol in vitro was studied by Lehman and Newman<sup>5</sup> in 1937 by a procedure similar to testing for red blood cell fragility. One part of rabbit blood was added to ten parts of mixed propylene glycol and water in which the glycol content did not exceed fifty per cent. Propylene glycol produced immediate hemolysis in all aqueous concentrations.

In commenting on these observations, Weatherby and Haag<sup>6</sup> concluded that aqueous solutions of propylene glycol produced rapid and complete hemolysis in a manner similar to that of distilled water. They assumed that the

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glycol entered the red blood cell membrane so rapidly that it exerted no appreciable osmotic effect on the cell. This explanation had been offered by Johnson, Carlson, and Johnson<sup>7</sup> for the apparently similar type of hemolytic phenomenon occurring with aqueous solutions of glycerol or urea.

*Behavior of Erythrocytes in Mixtures of Propylene Glycol and Distilled Water.*—Procedure.—Propylene glycol and distilled water were mixed in various proportions in a series of test tubes maintained at 0°, 20°, 37°, and 50° C. The first tube of each series contained only distilled water, the second, 10 per cent glycol, and the succeeding tubes contained increasing amounts of propylene in 10 per cent increments. One part of human venous blood was added to twenty parts of each solution; the tubes were inverted to mix, corked, and observed at intervals for a period of four days.

TABLE I

TIME OF FADING OF ERYTHROCYTES FROM MICROSCOPIC FIELD IN AQUEOUS CONCENTRATIONS OF PROPYLENE GLYCOL; COINCIDENT WITH CHANGE OF SOLUTION FROM TURBID TO CLEAR

PER CENT PROPYLENE GLYCOL IN WATER	TEMPERATURE			
	0° c.	20° c.	37° c.	50° c.
100	$\frac{1}{2}$ min.	—immediate—		
90	$1\frac{1}{2}$ min.	1 min.	$\frac{1}{2}$ min.	$\frac{1}{2}$ min.
80	$\frac{1}{2}$ min.	2 min.	$\frac{1}{2}$ min.	$\frac{1}{2}$ min.
70	20 min.	$2\frac{1}{2}$ min.	1 min.	1 min.
60	22 min.	3 min.	1 min.	1 min.
50	25 min.	3 min.	$1\frac{1}{2}$ min.	$1\frac{1}{2}$ min.
40	25 min.	8 min.	$1\frac{1}{2}$ min.	$1\frac{1}{2}$ min.
30	25 min.	150 min.	$1\frac{1}{2}$ min.	$1\frac{1}{2}$ min.
20	22 min.	8 min.	$1\frac{1}{2}$ min.	$1\frac{1}{2}$ min.
10	5 min.	2 min.	1 min.	1 min.
0	2 min.	$1\frac{1}{2}$ min.	1 min.	1 min.

*Macroscopic Observations.*—Three stages in the gross appearance of the blood-propylene glycol-aqueous mixtures were observed. The solutions were first turbid. This stage was followed by a gradual clearing until the solutions became transparent, suggesting complete hemolysis. The time required for the transition from turbidity to translucency (Table I) depended upon the temperature and glycol concentrations; it occurred more rapidly at higher temperatures and at both extremes of propylene glycol concentration. The longest interval was noted with 30 per cent glycol at 20° C. The third and final stage was characterized by the redevelopment of a turbid solution due to the formation of a reddish-brown flocculent precipitate. Von Oettingen and Jirouch<sup>4</sup> reported the formation of a similar precipitate with glycols although they did not use propylene glycol. As shown in Table II, the final precipitate developed only with higher temperatures and with higher concentrations of glycol; the time required for beginning precipitation varied with the temperature and glycol content. Except with one hundred per cent glycol, the formation of the precipitate was a gradual process. In the course of the reaction progressively larger amounts of precipitate were obtained as measured by centrifugation until an end point was reached, after which the amount of precipitate decreased and the supernatant fluid became clear. In later studies similar results

were obtained by measuring the amounts of precipitate by means of a photo-electric colorimeter.

A similar precipitate developed when erythrocytes, thrice washed and resuspended in saline, were added to 60 per cent and higher concentrations of propylene glycol in water. Cell-free plasma also precipitated when added to the same concentration of propylene glycol. From these observations, it appeared that the reaction giving rise to flocculation and precipitation might be one affecting all blood proteins and might not be limited to the erythrocytes.

*Microscopic Observations.*—The mixture of blood in 80 per cent propylene glycol in water was selected for detailed microscopic observation, because at this concentration it was possible to observe the three phases of the reaction within a relatively short time. As viewed under oil immersion, the erythrocytes faded in two minutes although many faintly visible and nonrefractile red cell forms remained; these were brought out more clearly by reducing the light or by making minor adjustments in the condenser. After about thirty minutes, the cells gradually became more distinct; with average illumination one obtained the impression that the red cells had "reappeared." With further time, many erythrocytes developed hairlike projections at right angles to the cell wall; each was associated with the development of an increasing amount of granular, extracellular debris. Spontaneous agglutination of these abnormal cells then occurred, giving rise to the macroscopic appearance of turbidity and flocculation. The individual cells of these agglutinated masses finally disintegrated, leaving only debris, this stage corresponding to the end point of macroscopic precipitation.

Similar microscopic changes in the erythrocytes occurred in other mixtures containing over sixty per cent propylene glycol, except that the rate of the reaction varied in proportion to the glycol content. In a concentration of 60 per cent glycol at 20° C., a few red cells remained refractile and clearly visible at the end of seven days; the number of such cells was apparently not sufficient to result in the gross appearance of turbidity. Agglutination and flocculation were not observed in this tube and did not occur in other tubes containing less amounts of propylene glycol. A microscopic study of the "ghost" forms in lower concentrations was not undertaken because of their relatively poor visibility as viewed in the conventional microscope.

With 30 per cent glycol and 70 per cent water at 20° C., the red cells, although enlarged, remained clearly visible for approximately two and one-half hours before fading.

*Dark-Field Observations.*—Blood was added to various concentrations of propylene glycol and examined immediately under the dark-field microscope. In an aqueous solution of 70 per cent propylene glycol at 20° C., the red cells became uniformly shrunken and rapidly crenated. Agglutination soon followed, and by the end of thirty minutes all erythrocytes had disintegrated. Red cells in 50 per cent propylene glycol appeared normal in size and contour two minutes after mixing. The cells gradually became shrunken and crenated; with the passage of time, many became agglutinated and some possessed spicules extending at right angles from the cell wall. Although distorted in form, the erythrocytes remained clearly visible for as long as ten days. With decreasing

concentrations of propylene glycol, a progressive swelling of erythrocytes was noted, eventually leading to the disruption of the cells.

*Behavior of Erythrocytes in Mixtures of Propylene Glycol and Physiologic Saline.*—Lehman and Newman<sup>5</sup> in 1937 produced hemoglobinuria in animals by the intravenous injection of a 33.3 per cent solution of propylene glycol in saline. They concluded that the hemoglobinuria resulted from the hemolysis of erythrocytes and was due to the osmotic action of glycol on the cells at the site of the injection. The authors further mixed one part rabbit blood with ten parts propylene glycol in saline solutions and observed no hemolysis in concentrations of 40 per cent propylene glycol and below at the end of three hours; in 40 per cent glycol, partial hemolysis was present at twenty-four hours. Immediate hemolysis was produced under the same conditions and concentrations by propylene glycol and water.

Similar findings were reported by Weatherby and Haag<sup>6</sup> with human blood in various concentrations of propylene glycol and physiologic saline. They felt that the absence of hemolysis in saline solutions of propylene glycol in this range was to be expected as a 30 per cent concentration was essentially an isotonic medium.

TABLE II

TIME OF "REAPPEARANCE" OF ERYTHROCYTES IN MICROSCOPIC FIELD IN AQUEOUS CONCENTRATIONS OF PROPYLENE GLYCOL; COINCIDENT WITH CHANGE OF SOLUTION FROM CLEAR TO TURBID AND APPEARANCE OF GROSS PRECIPITATE

PER CENT PROPYLENE GLYCOL IN WATER	TEMPERATURE			
	0° c.	20° c.	37° c.	50° c.
100	1 min.	immediate		
90	1,200 min.	4 min.	3 min.	1 min.
80		40 min.	5 min.	2 min.
70		600 min.	20 min.	3½ min.
60			150 min.	4½ min.
50			2,400 min.	5 min.
40				10 min.
30				25 min.
20				80 min.
10				180 min.
0				-----

Present observations on whole human blood in saline concentrations of propylene glycol confirm the reports of previous authors. Twenty-four hours were required for the apparent hemolysis of solutions containing 40 per cent glycol and forty hours for 30 per cent solutions. The red cells remained intact and the solutions remained turbid for as long as seven days in concentrations of propylene glycol below 30 per cent. In amounts of 60 per cent glycol and above, the clear solutions later became turbid and developed a reddish brown precipitate. This phenomenon occurred more rapidly in higher concentrations of propylene glycol and in all respects appeared similar to the reaction occurring with aqueous solutions. At a time when the gross appearance of the solution suggested complete hemolysis, microscopic examination revealed the faded "ghost" forms previously described, but the red cells remained clearly visible and in normal numbers when observed under dark-field illumination.

*The Action of Other Glycols on Erythrocytes in Vitro.*—The action of ethylene glycol and its derivatives on blood was studied by Von Oettingen and Jirouch.<sup>4</sup> They added 1 c.c. of various glycols to 5 c.c. fresh dog blood or beef serum; ethylene and diethylene glycol failed to precipitate whereas ethyl ethylene glycol acetate, one to four dioxan and butyl ethylene glycol caused marked precipitation.

In the present study aqueous solutions of ethylene and triethylene glycol with human blood were observed in the proportions previously described. Except with 30 per cent concentrations where the time required for the solutions to clear was prolonged, all other concentrations of both glycols resulted in the gross appearance of hemolysis within fifteen minutes. The speed of the clearing of the reaction was more prolonged at room temperature than was the case with propylene glycol. In higher concentrations, both ethylene and triethylene glycol produced a similar type of reddish-brown precipitate.

*Discussion.*—Erythrocytes are visible in the conventional bright field microscope because of their ability to refract light. This in turn is dependent upon a difference in the relative densities of the cells and the surrounding medium. When erythrocytes are suspended in solutions of over 30 per cent propylene glycol in water, the density of the red cell apparently approaches that of the solution. Light rays may then pass from one to the other without significant bending, with the result that the erythrocytes become relatively invisible in the conventional microscope, although clearly outlined in the dark field, and the gross appearance of the suspension changes from one of turbidity to clearness.

The "fading" of erythrocytes in the presence of less than 30 per cent propylene glycol in water appears to be primarily the result of hemolysis, as evidenced by progressive swelling of the cells prior to their disappearance. The rate of "fading" is, furthermore, roughly proportional to the hypotonicity of the mixture, and relatively few intact cells are seen when the clear solutions are viewed in the dark-field microscope.

Thirty per cent propylene glycol and 70 per cent water apparently represent a critical glycol concentration, above which the red cells are affected primarily by glycol and secondarily by water, and below which primarily by water and secondarily by the glycol content. The marked delay in the "fading" of erythrocytes in this concentration at 20° C. is very likely due to the relatively ineffective glycol action at this temperature and dilution and the mild hypotonicity of the solution.

The spontaneous reappearance of red cells at higher temperatures and glycol concentrations is presumably the result of an increase in the intracellular versus the extracellular density and is most probably associated with the coagulation or precipitation or other alteration of cellular proteins, a process which finally leads to cell destruction and the formation of a dark granular precipitate. Alteration of cell-free plasma also occurs in the presence of higher concentrations of propylene glycol, resulting in precipitate formation; this suggests that the physiochemical reaction produced by propylene glycol is not one specifically affecting erythrocytes.

In concentrations above 30 per cent propylene glycol, erythrocytes behave similarly whether suspended in glycol-aqueous or glycol-saline solutions. Al-

though the "fading," "reappearance," and final disintegration of cells develop at different rates with alterations of the temperature and glycol concentration, at a given temperature and content of glycol the effects occur much more slowly in the presence of saline. The retardation becomes more marked as the glycol percentage is decreased and that of saline increased; for instance, at 40 per cent and 30 per cent glycol in saline, twenty-four and forty hours are required for the "fading" of erythrocytes from the bright field.

There is a fundamental difference in the behavior of red cells in concentrations below 30 per cent propylene glycol according to whether the solutions contain saline or water. In the saline, the red cells remain intact and clearly visible for many days. The presence of more than 70 per cent physiologic saline appears to protect the red cells from the action of glycol. In the water, the erythrocytes "fade" roughly in proportion to the hypotonicity of the solution.

Ethylene and triethylene glycol exert essentially similar effects on erythrocytes as produced by propylene glycol.

*Summary.*—1. Observations are reported on erythrocytes suspended in aqueous and saline concentrations of propylene and other glycols.

2. The erythrocytes were found at first to disappear from the microscopic bright field but not from the dark field, coincident with the apparent gross "hemolysis" of the blood-glycol solution.

3. Evidence is offered refuting the suggestion that hemolysis was the cause of the initial fading and disappearance of erythrocytes.

4. In time, depending on temperature and glycol concentration, the erythrocytes reappeared in the microscopic bright field.

5. The nature of the reaction between glycols and erythrocytes is considered.

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## THE EFFECT IN VITRO OF PROPYLENE GLYCOL ON LEUCOCYTES\*

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THE observation that leucocytes remain intact and clearly visible when suspended in equal parts of propylene glycol and distilled water led one of us (T. G. R.) to use this solution as a white blood cell diluting fluid.<sup>1, 2</sup> In comparison with the standard 2 per cent acetic acid diluting fluid, the glycol diluent possesses several advantages inherent in its greater viscosity, facilitating filling the counting chamber and decreasing the subsequent rate of evaporation. The incorporation of acid and basic stains, readily soluble in propylene glycol, further permits the differential staining of eosinophils, polymorphonuclear and mononuclear cells in the counting chamber. It is therefore possible to obtain a differential white cell count in the counting chamber on the same sample of blood used to enumerate the total leucocytes. Mononuclear cells include both lymphocytes, monocytes, and nonsegmented members of the polymorphonuclear series. Basophils are not differentiated from other polymorphonuclear cells. Eosinophils are stained characteristically, thus enabling their direct enumeration free from the errors of distribution inherent in the procedure of preparing and counting the Wright-stained film. The performance of a counting chamber differential in conjunction with the differential white count from the stained film gave additional evidence as to the identity of "smudges" encountered occasionally in films. The blood of twenty normal subjects was studied in the preliminary report, and this diluent yielded accurate total leucocyte counts when compared with parallel determinations with 2 per cent acetic acid diluting fluid. Counting chamber differential counts were also in good agreement with differential counts performed from the Wright-stained film.

The further use of stains dissolved in 50 per cent aqueous propylene glycol has confirmed the conclusions drawn in the preliminary study. It has been observed that blood diluted with this fluid may be allowed to stand in the pipette overnight without significant change in the total white count or impairment in the staining qualities of the leucocytes.

*I. Observations on Normal White Blood Cells.*—In a further study of leucocytes, one part of whole human venous blood was added to twenty parts of propylene glycol in distilled water. The mixtures were placed in a series of ten tubes, the glycol content of which varied by increments of 10 per cent; the first tube contained 10 per cent glycol and 90 per cent water, the final tube, 100 per cent propylene glycol. Each tube was corked, maintained at 20° C., and, prior to withdrawing samples for counting chamber study, was shaken gently. Observations were made on fresh samples at intervals of thirty minutes for the first three hours and every twelve hours thereafter for sixty

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hours. White cells were nonmotile in all samples. Eosinophils were not identified in the absence of stains, although mononuclears could be differentiated from polymorphonuclear leucocytes.

In a concentration of 60 per cent propylene glycol in distilled water the white cells remained for as long as sixty hours without apparent change. This concentration of glycol was found to represent a critical point, for with lesser amounts of glycol, the white blood cells became progressively enlarged, irregular, and less clearly outlined; the rate and degree of these changes varied inversely with the content of propylene glycol. At 50 per cent, 40 per cent, and 30 per cent propylene glycol, the mononuclear cells were the first to become enlarged and irregular, followed by similar changes in the polymorphonuclear leucocytes; the changes in both types of cells occurred more rapidly with progressive dilutions. Cells in 50 per cent solutions showed slight changes, but remained clearly visible and at a constant numerical level for as long as sixty hours. In 40 per cent solutions, the leucocytes showed more marked abnormalities in their size, shape, and refractility; at the end of two hours, distortion and fading interfered with their enumeration. In 30 per cent and lower concentrations of propylene glycol, the above changes occurred immediately and were followed by a tendency of the leucocytes to agglutinate. The initial white cell count at this concentration was below normal and with serial determinations continued to fall.

At concentrations above 60 per cent propylene glycol, the leucocytes became smaller in size. In a 70 per cent solution, the white cells remained clearly visible for two hours; after this time the gradual "reappearance" of erythrocytes interfered with the enumeration of leucocytes. At concentrations above 70 per cent, this "reappearance phenomenon" occurred too rapidly to allow the initial enumeration of white cells, although the latter remained visible in concentrations of 90 per cent and 100 per cent propylene glycol. The phenomenon of "reappearance" of erythrocytes in higher glycol concentrations has been described.<sup>3</sup>

In an attempt to determine the range of propylene glycol concentrations in which the size and shape of white cells remained unaltered, blood was added similarly to mixtures of 58 per cent and 62 per cent glycol in water. In the lower concentration mononuclear cells became slightly enlarged at the end of thirty minutes and polymorphonuclear cells became distorted at the end of two hours. In 62 per cent glycol all white blood cells became uniformly small but remained refractile and clearly visible for sixty hours.

*Differential Staining.*—As previously described, eosin and methylene blue each as a 0.1 per cent aqueous solution in 50 per cent propylene glycol produced a satisfactory stain for the leucocytes with a minimum of distortion. Blood placed in such a solution was studied under high magnification. Within a five-minute period the granules of the eosinophil stained a bright red. The capsule became progressively and uniformly enlarged until it finally appeared as an eccentric swelling. The intervening space between the capsule and the cytoplasm remained clear except for an occasional granule observed on the capsular rim. The eccentric arc-like enlargement of the capsule frequently reached a size twice that of the parent eosinophil; it was occasionally seen to rupture



although this did not occur within the first thirty minutes. After rupture of the membrane the cell remained intact, spherical in outline, and surrounded by a small concentric capsular ring.

Polymorphonuclear neutrophils in 50 per cent propylene glycol showed similar but less frequent and extensive changes. Capsular swelling was usually concentric and only detectable under oil immersion. Eccentric swelling was seen rarely in normal blood nor was rupture of the capsule observed. As with eosinophils, capsular swelling of neutrophils occurred more readily at lower concentrations of glycol and was not detected in 60 per cent propylene glycol in water.

Mononuclear cells did not show a capsular swelling. However, a uniform enlargement of the cell was seen in 50 per cent but not in 60 per cent propylene glycol.

All leucocytes were more deeply stained in concentrations of propylene glycol below 50 per cent, but the distortion of the cells in these aqueous concentrations did not favor their use. The staining qualities were distinctly inferior at 55 per cent and 60 per cent propylene; at higher strengths the cells failed to stain.

TABLE I

COMPARISON OF THE TOTAL LEUCOCYTE COUNTS USING 2 PER CENT ACETIC ACID AND PROPYLENE GLYCOL-AQUEOUS STAINS

PATIENT	DIAGNOSIS	TOTAL WHITE CELL COUNT	
		PROPYLENE GLYCOL-AQUEOUS STAIN	2 PER CENT ACETIC ACID
L. H.	Lymphoblastic Leucemia	108,000	102,000
E. M.	Lymphoblastic Leucemia	127,000	123,000
F. T.	Lymphoblastic Leucemia	300	300
F. H.	Chronic Lymphatic Leucemia	31,000	27,000
W. G.	Lymphoblastoma	35,800	33,850
U. S.	Myelogenous Leucemia	319,000	292,000
M. S.	Myelogenous Leucemia	282,500	266,500
W. M.	Myelogenous Leucemia	45,000	40,500
F. M.	Myelogenous Leucemia	130,000	130,000
B. B.	Monoblastic Leucemia	170,000	195,000
F. S.	Monocytic Leucemia	92,350	87,500
W. B.	Histio-monocytic Leucemia	32,500	35,000
E. P.	Aluecemic Leucemia	2,900	3,500

The propylene glycol diluent consisted of equal parts of the two stock solutions. Maximum staining qualities were observed when the diluent was prepared from the stock each day.

Solution I  
Methylene blue 0.1 Gm.  
Propylene glycol 100 c.c.

Solution II  
Eosin B 0.1 Gm.  
Distilled water 100 c.c.

II. *Observations on Abnormal White Blood Cells.*—It became of interest to study the blood of patients with leucemia and with conditions characterized by the presence of abnormal or immature leucocytes. Table I records parallel total white blood cell determinations made by the standard 2 per cent acetic acid diluent and with the propylene glycol aqueous stain.

In the case of L. H. in the five days following irradiation therapy, the total leucocyte count increased from 6,700 to over 100,000 per cmm.; and a differential count made with the Wright-stained cover slip method revealed 92 per cent "smudges." Despite the apparent fragility of the white cells, the total leucocytes determined with propylene glycol agreed closely with that obtained

with 2 per cent acetic acid. The differential count from the Wright-stained cover slip revealed 3 per cent polymorphonuclear cells, 1 per cent eosinophils, 4 per cent large lymphocytes, and 92 per cent "smudges." A parallel counting chamber differential count identified the "smudge" forms as a mononuclear type cell with a differential showing 4 per cent polymorphonuclear cells, 1 per cent eosinophils, and 95 per cent mononuclear cells.

Propylene glycol and water in equal parts appears to be a satisfactory diluent for counting abnormal white blood cells in leucemia.

*Discussion.*—Enlargement of the capsules of eosinophils and polymorphonuclear leucocytes and a uniform enlargement of mononuclear cells occur in the presence of equal parts propylene glycol and water. This does not invalidate the use of this diluent for determining the total white cell count with pathologic cells as encountered in leucemia.

Satisfactory differential staining of leucocytes with eosin and methylene blue occurs only when the diluent consists of equal parts of propylene glycol and water. Optimum staining with minimum cell distortion occurs in 50 per cent propylene glycol in water, for with lower concentrations the cells become distorted in outline and visibility despite adequate staining. With 60 per cent propylene glycol staining is unsatisfactory, although no change in the size of the cells occurs for long periods of time.

*Summary.*—1. A white blood cell diluting fluid consisting of eosin 0.1 per cent and methylene blue 0.1 per cent dissolved in equal parts propylene glycol and water has been found to give accurate leucocyte counts in normal and abnormal blood.

2. This diluent possesses certain technical advantages over the use of the standard acetic acid diluting fluid.

3. The effect of various aqueous concentrations of propylene glycol on leucocytes in vitro has been described.

We wish to acknowledge the technical assistance of Elizabeth Barber Gibson and the valuable suggestions of Don E. Francke.

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## A STUDY OF THE PAPPENHEIM STAIN—A STABLE MODIFICATION\*

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PAPPENHEIM'S compound stain could be a useful aid to the bacteriologist for the examination of bacteria in pus if it were more stable. The differential qualities and the higher percentage of positive findings make further attempts at increasing its stability worth while.

Pappenheim<sup>1</sup> prepared his stain from solid or concentrated aqueous solutions of methyl green and pyronine in a study of lymphocytes and plasma cells. Unna<sup>4</sup> preferred a stock reagent of the two dyes in alcohol-glycerin solution with 0.5 per cent phenol added. Krzysztalowicz<sup>5</sup> employed 2 per cent phenol in the Unna reagent to decrease staining reaction time of gonococci in smears of exudates. Saathoff<sup>6</sup> used higher concentrations of pyronine and alcohol as well as 2 per cent phenol in his stain for gonococci in pus smears. Conn et al.<sup>7</sup> suggested the use of pyronin "B" in place of pyronin "G." Pappenheim<sup>3</sup> and Scudder<sup>8</sup> advocated the use of the Pappenheim reagent as a counterstain in the Gram reaction. Walton<sup>9</sup> suggested the use of methyl alcohol in place of ethyl alcohol.

Our previous efforts to employ the Pappenheim stain or its modifications were disappointing due principally because of the instability of the methyl green and the variation of dyes on the market. Freshly prepared products were satisfactory in our hands, but when reagents were given to others, indifferent results were obtained by them. Several experiences in our attempts to prepare a satisfactory Pappenheim stain may be of sufficient interest to record here.

A 1 per cent solution of methyl green in distilled water and a 1 per cent solution of methyl green in 2 per cent aqueous phenol were prepared and tested using the filter paper spot test.†

The methyl green in distilled water dissolved completely, and the spot formed on filter paper was green with a distinct blue or purple center. The methyl green in 2 per cent aqueous phenol, however, yielded a precipitate, and the supernatant fluid produced a green spot on filter paper without a trace of purple or violet. The precipitate formed in aqueous phenol was crystal violet.

A similar precipitate may be produced by adding phenol to aqueous crystal violet solutions, the degree of precipitation being a function of the concentration of phenol.

\*From the Snodgrass Laboratory of Pathology and Bacteriology, City Hospital. Received for publication, July 24, 1913.

†The filter paper spot test was employed by Pappenheim<sup>1</sup> as stain reagents. A drop of stain was placed on a filter paper periphery of the spot showed the relative amount of green in : showed the amount of red. Using this test one could estimate both methyl green and pyronine or the concentration of crystal green stock solutions. One may determine roughly how much to the other or with water. This test has been useful, but not all equal diffusion through filter paper can be used in Pappenheim compound stains with success.

Pappenheim reagents prepared as described by Saathoff and by Walton (both modifications contained 2 per cent phenol) produced a precipitate of crystal violet which formed a gummy residue on the glassware and filter. Hence, the phenol served to remove by precipitation the crystal violet which was contained as an impurity in the commercial methyl green dye.\*

Stain reagents prepared according to Walton's formula, or prepared from stock aqueous solutions of purified methyl green and pyronine, gave suitable stains when freshly prepared. In our hands, however, the methyl green continued to split off crystal violet. Walton<sup>9</sup> reasoned that methanol, substituted for ethanol, would suppress the dissociation of the loosely bound methyl group of methyl green and retard the formation of crystal violet. This might be the case if the methyl green were dissolved in aqueous methyl alcohol alone, but it would seem that inhibition of the crystal violet formation would be very slight in the Walton formula, since the phenol present would precipitate the crystal violet and encourage the reaction (methyl green  $\rightarrow$  crystal violet  $\downarrow$  +  $\text{CH}_3\text{OH}$ ) to go to completion. To employ a reagent which would not precipitate the crystal violet would impair the differential character of the stain, as crystal violet stained gonococci and leucocytes and debris with equal intensity.

Pyronine seemed to have an unusual affinity for the Neisserian organisms. It was stable under a wide range of conditions and its use in the stain offered no great difficulty. The need was for a stable dye of contrasting color which would stain the nucleus of a leucocyte intensely, the cytoplasm of the cell poorly, and the intracellular bacteria still less or not at all. In looking for dyes that might take the place of methyl green, we examined the blue and green basic dyes that are commonly employed in bacteriologic laboratories. Two such dyes were brilliant green and malachite green. Both dyes when used in combination with pyronine gave a filter paper spot test as good as that obtained with a good, fresh methyl green reagent.† Although both of these basic green dyes were described by Pappenheim<sup>2</sup> as unsuitable for combination with pyronine in his differential stain, it has been our experience that either may be utilized satisfactorily for the production of a stable Pappenheim-type stain. It has seemed that malachite green produced a slightly better product, but either dye gave satisfactory color contrast.

The following formula is suggested as a guide in the preparation of a stable modification of the Pappenheim reagent giving good differential stains:

1 per cent aqueous malachite green (oxalate, certified "NMg-3,"	
dye content 75 per cent -----	1.2 c.c.
0.5 per cent aqueous pyronine G (certified "NP-7," dye content high,	
but not specified on label) or pyronine 2G (Coleman and Bell) --	3 c.c.
Distilled water -----	20 c.c.

Prepare stock solution of malachite green in distilled water two days before use and filter. Stain from thirty seconds to three minutes. Wash with water.

\*Conn<sup>1</sup> suggested that methyl green may be extracted with chloroform or amyl alcohol to remove the crystal violet impurity. Chloroform extraction has markedly improved the lot of methyl green with which we worked.

†Walton kindly supplied a sample of his reagent.

A good stain is produced by a delicate balance of the concentrations of the two dyes and of the time and temperature of their reaction on the tissues. The two dyes (the green and the red) must be balanced in respect to their affinity to the tissue and their color intensity. Thick smears stained deeper than thin smears, but differentiation was usually better in the thick portions of the preparations. The time of staining was important, for in overstained smears gonococci were dark, having taken some of the green color, and the contrast was poorer than in preparations stained for shorter periods.

It is suggested, in view of the variations in dye content of products on the market and in view of the preference of individuals using the stains, that stock solutions of each of the two dyes be prepared and several reagents be formulated in test tubes for trial, varying the ratio of the dyes until a product is obtained which gives a differentiation and contrast satisfactory to the user. The best reagent may then be prepared in larger volume and stored for use.

It should be noted especially that buffer salts of pH 4.7 or more precipitated and decolorized dilute solutions of brilliant green and malachite green, a change that did not take place at pH 4.5 or less. The tap water (pH 8.2) in our laboratory caused a precipitation and fading of these dyes. Some specimens of "distilled" water likewise caused slight changes in color intensity, but tests indicated that these samples were contaminated with alkaline substances carried over in the distillation. Dilute solutions of the dyes in distilled water of a good quality did not fade. It is possible that Pappenheim may have encountered similar conditions in his trial of these two dyes.

Because of the effect of alkaline salts on brilliant green and malachite green, aqueous solutions have been used employing *distilled* water. It has not been found necessary to incorporate glycerol, phenol, ethanol, or methanol in this modified stain. Pyrex glassware has been used for storage of the stock solutions to avoid the alkali of ordinary glass containers.

Freshly prepared solutions of malachite green may stain more intensely than those two or more days old. Aside from this initial change, stain reagents and stock solutions of the dyes have shown no depreciation in staining qualities during two years of storage at laboratory temperature on a shelf exposed to daylight. New stain reagents prepared from old or new stock solutions of malachite green and pyronine G were indistinguishable from old stain reagents.

Several serious limitations of the Pappenheim stain have been noted. Very thin smears were apt to be faintly colored even with a more extensive period of staining. Although these could still be examined microscopically and contrast was sufficient for observation of phagocytized organisms, they were more difficult to observe. The use of heavier smears (for which the Pappenheim stain is eminently suited) should remedy this situation.

A second limitation of the stain is less serious. The smears are best stained when treated individually. The separate handling of the slide preparations is offset by the simplicity and the rapidity of the staining procedure.

We can confirm Walton's statement that positive reports may result more frequently from the use of this stain in examination of gonorrheal exudates than from the exclusive use of the Gram stain. In some instances we have found typical organisms with relative ease in a Pappenheim-stained preparation, whereas the Gram-stained slide showed numerous leucocytes or much debris with-

out distinguishable bacteria of any kind. This was particularly true in heavy prostatic smears or in specimens containing considerable blood. We do not feel able to differentiate between gram-positive and gram-negative diplococci using the Pappenheim stain alone. We prefer to employ the reagent as a counterstain for the Gram reaction (as suggested by Pappenheim<sup>3</sup> and more recently by Scudder<sup>8</sup>), although this decreased the brilliance of the stain of the bacteria in the smear and it was necessary to shorten materially the staining time of the Pappenheim reagent.

The stain is useful in the examination of pus aspirated from chancroidal buboes, since the occasional *H. ducreyi* is so much more readily seen than with the Gram stain. Cultures of the Ducrey bacillus in the clotted rabbit blood described by Teague and Deibert<sup>10</sup> are likewise more easily examined when stained by the Pappenheim stain. The young Ducrey organisms stain intensely with the pyronine against a green background.

Many bacteriologists doubtless remember their first encounter with the Gram reaction and the dissatisfaction expressed by beginning students in bacteriology when first attempting to employ the stain. Experience and judgment are just as necessary in employing and interpreting results obtained with the Pappenheim stain. It is not believed that a stable Pappenheim-type stain will replace the Gram stain in routine microscopic examination of gonorrheal exudates, but a dependable product would undoubtedly be more generally employed to augment the staining procedures now used.

*Summary.*—A stain of the Pappenheim bicolor type which is stable and which gives as good differentiation as the original freshly prepared methyl green pyronine reagent is described.

The stain is of special value in the examination of bloody specimens, heavy smears of exudates, and blood clot cultures of chancroidal pus. It should be a valuable supplement to the Gram stain.

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## ON THE CARE OF HUMAN SPERM\*

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THE early reports concerning in vitro care of sperm were ubiquitous and inadequate. Since the study of sperm physiology and vitality has more recently been undertaken, their per se preservation has become fundamental. Furthermore, lack of ready availability of human sperm makes it more imperative that any test undertaken gives the desired results without repetition.

If the desired test is for survival time of spermatozoa or for artificial insemination, the donor should provide the ejaculate while at the office. The ejaculate should be collected directly into a chemically clean, sterile receptacle. A calibrated centrifuge tube serves well.

### THE EFFECT OF RUBBER, OF BRASS, AND OF COPPER

Human ejaculates for study purposes are still frequently and erroneously collected from condoms. Rubber, even on short time exposure, decreases markedly the duration of motility of spermatozoa. Brown and Gamble (1943) report the results of several tests in which the ejaculate was divided, the one fraction used as a control and the other exposed to rubber, i.e., condoms and eye dropper bulbs. Some of the condoms had been thoroughly washed in tap water to remove the powder; others were used as packed. The age of spermatozoa exposed for only five minutes was reduced to 60 to 70 per cent of control time; with constant exposure, the spermatozoa had only 25 per cent of the control life span. The time difference with exposure to the unwashed condom was negligible. Constant exposure to brass, which may be encountered in lids, caps, etc., placed on a vial containing the ejaculate, decreased the longevity to approximately 30 per cent of the control. Copper essentially duplicated this effect.

### THE EFFECT OF AIR AND TEMPERATURE

Several investigators have reported that air affects spermatozoa variously. In a series of experiments Brown and Gamble (1943) determined that there was no appreciable difference between the duration of motility of the control and sperm which had air excluded by an overlay of mineral oil. The effect of temperature, on the other hand, is pronounced. The average duration of locomotion in 14 experiments with an initial average spermicidal time of approximately one hundred minutes with 2.8 per cent potassium acid phthalate solution was 20 to 25 hours at body temperature (37° C.), 70 to 75 hours at

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†The author has observed several instances in which a leucocyte had ingested an active spermatozoon; pronounced flagellation ensued and continued for several minutes.

room temperature (approximately 27° C.), and 140 to 150 hours at refrigerator temperature (5° C.). The 2.8 per cent phthalate was mixed with an equal amount of semen, and the immobilization time (spermicidal time) was noted (Brown and Gamble, 1940, 1941). This was repeated at convenient intervals until the spermicidal time reached two to seven minutes; this was called the aging time or longevity (Brown and Gamble, 1941, 1943).

#### EFFECT OF VAGINAL SECRETION, URINE, PUS, AND BACTERIA

Brown (1943) determined that the longevity time of spermatozoa in a mixture of equal parts semen and vaginal secretion was reduced to 55 to 60 per cent of control time. Spermatozoa from the postcoital vagina, being constantly in contact with intermixed secretion, are inferior to the control. Urine which was normal by routine laboratory test reduced the longevity time to about 60 per cent of control time; diabetic urine (3+), to 80 per cent; and ieteric urine (3+), to about 45 per cent (Brown, 1943). Pus from urethritis, etc., decreased the longevity variably depending on the degree of purulency. Pus cells may ingest the spermatozoa (3), and fragments and cells at times adhere to the flagellum, etc., of the spermatozoon, thereby decreasing its activity. Spermatozoa contaminated with bacteria invariably have a decreased longevity, probably from toxic metabolites. Motile bacilli may also be confusing to the microscopist when using low power oblique dark-field illumination.

#### ESSENTIAL CONTROLS

Because of the pronounced variation of spermatozoal vitality as determined by spermicidal times (Brown and Gamble, 1940, 1941, 1943), controls to determine the vitality and aging process of spermatozoa are essential at the outset and during tests.

#### SUMMARY

1. Sperm should be collected directly into clean glass vials.
2. It should not contact rubber condoms, stoppers, or bulbs even for a short interval. Contact with brass and copper should also be avoided.
3. Contact with air has no demonstrable effect. Temperature should be kept constant during an experiment as variations affect the spermatozoal longevity. Sperm should be preserved at temperatures approximating 5° C. for reduced aging effect.
4. Sperm collected from the vagina age more rapidly than the control. Urine, pus, and bacteria are deleterious.
5. Carefully planned controls should be run concurrently with all tests on spermatozoon vitality.

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## A SIMPLE APPARATUS FOR DETERMINING PERIPHERAL ARTERIAL PRESSURE IN THE RABBIT'S EAR\*

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THE apparatus described below is designed to determine the pressure of the central artery of the rabbit's ear. Devices similar in principle have been described, notably that of Grant and Rothschild (1934). However, in our hands results with this simpler apparatus have been uniform when the indicated precautions were observed.

A glass reservoir (Fig. 1) (A) containing mercury is connected by means of rubber tubing with a one-half inch T tube (B). The lower arm of the T (C) contains a lucite rod of proper length which serves to transmit light from the source (D) and also to provide a seal for the lower portion of the air chamber. The open end of the upper arm of the T is covered by a small sheet of condom rubber which is securely fastened around the neck of the tube. Care must be taken not to stretch the rubber while fastening in place. Over the top of the rubber-covered chamber is placed a plate of glass (E), about 1 inch by 4 inches by  $\frac{1}{4}$  inch. This glass is attached to a screw (F) so that it may be moved freely up and down over the air chamber. When in use, the shaved ear of the rabbit (the animal anesthetized or held in a suitable holder) is permitted to rest by its own weight on the rubber-covered top of the air chamber. The glass plate is then screwed down over the ear, holding it firmly but gently enough not to compress the artery. By observing from above, the position of the ear is easily adjusted so that the artery lies across the diameter of the chamber tube. A binocular dissecting microscope (25 diameters) supported above the plate (E) facilitates adjustment and observation of the artery. By means of the needle valve (G) the zero point is adjusted on the mercury manometer (H). The mercury bulb is then raised by means of a suitable screw and ratchet, which balloons out the rubber diaphragm covering the inflation chamber and compresses the artery between it and the glass plate above. When the artery is seen to be just occluded the manometer is read. By carefully adjusting the occluding pressure it is possible to measure both diastolic and systolic pressures. Several readings on one animal may thus be taken in rapid succession, but it is essential to take successive readings on the same point on the artery. If these readings diverge greatly it may indicate leakage or other defects in the apparatus, provided both the environmental and animal temperatures remained constant and the animal was not restless.

The apparatus is constructed of  $\frac{3}{8}$  inch pressure tubing, and great care must be taken to see that all connections are airtight. The most frequent point of leakage is the rubber diaphragm. It is desirable to check this each time the instrument is used. The whole should be suitably supported and firmly fastened to the table top.

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A suitable rabbit holder may be made by shaping plaster of paris bandages about a model which is then cut into an upper and lower section. A rabbit placed in a crouching position in the lower half is held firmly but in a comfortable position by strapping down the upper section. The ears protrude through an opening in the top. The rabbit quickly learns to remain perfectly quiet for the short period necessary to obtain manometer readings.

The apparatus has also been used successfully with guinea pigs and rats.

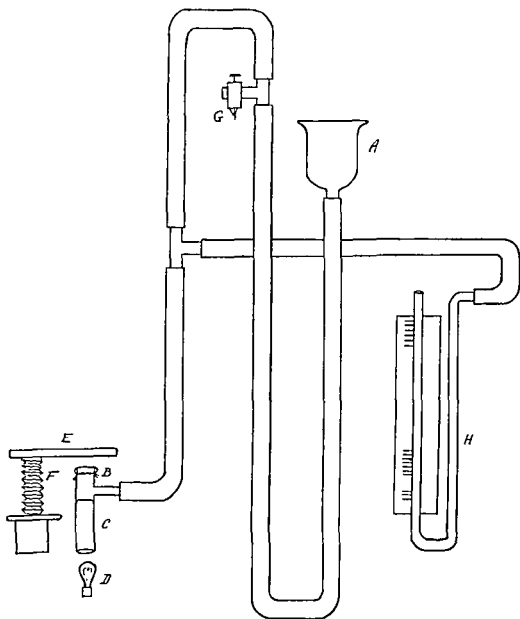


Fig. 1.—Explanation in text

Although the readings obtained with this apparatus were found to be consistent and reproducible under controlled conditions, it was desirable to compare the readings on the peripheral pressure so obtained with the systemic arterial pressure as obtained through the femoral or carotid arteries. This procedure was also expected to serve as an accurate check on the efficiency of the apparatus. Several rabbits were tested in this way. On the anesthetized animal, a carotid cannula was inserted and connected with a *U* tube mercury manometer. Simultaneously readings were taken on the ear artery with the apparatus described above. The two pressures were recorded at the same moment by different observers. Readings were first taken on the anesthetized animal at about one minute intervals for fifteen to thirty minutes. Later the animal

TABLE I

SIMULTANEOUS READINGS ON CAROTID AND PERIPHERAL (EAR) ARTERIAL PRESSURES, IN MM. OF MERCURY. RABBIT. ANIMAL NO. 10

TIME	CAROTID	PERIPHERAL
2.05	68	22
2.06	70.5	23
2.07	63.5	19
2.08	67	20
2.10	67	19.5
2.11	70	21.5
2.12	67.5	21
2.14	69	21
2.15	69.5	21.5
2.16	68	20
2.17	71	21.5
2.19	68.5	19
2.21	71	21
2.22	69	20
2.23	68	19
2.24	67.5	17
2.25	68	18.5
2.26	67	17
2.27	67.5	18
2.28	69	20.5
2.29	69.5	21
2.31	69	20.5
2.32	70	21.5
2.33	69.5	21
2.34	68.5	19
2.35	69	20
2.37	69	20.5

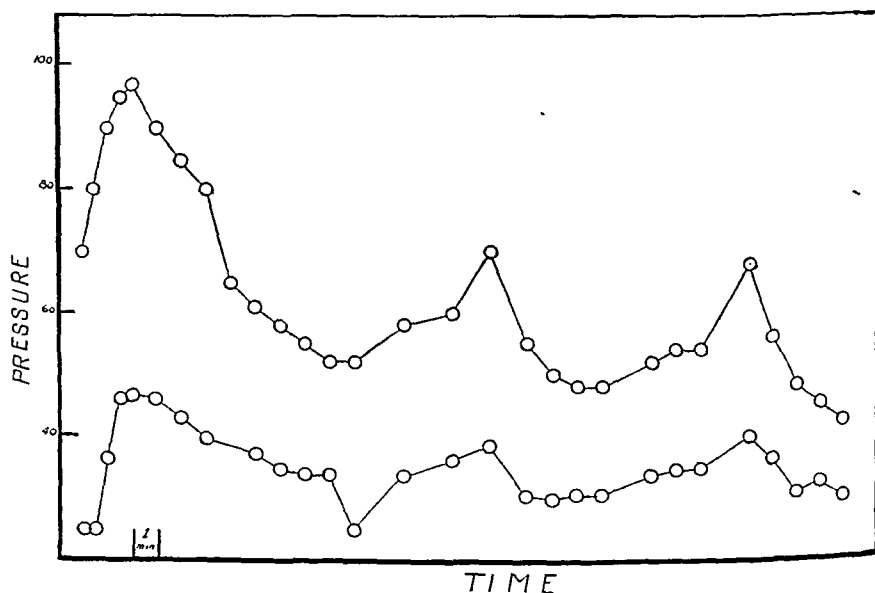


Fig. 2.—Pressure of carotid artery (upper curve), central artery of ear (lower curve), in mm. of mercury. Simultaneous readings. 1.1 c.c. pitressin intravenously one minute before first reading. Rabbit. Animal No. 14.

received pitressin intravenously and both carotid and peripheral pressures were again recorded. Table I gives the data on these readings over a thirty-five minute period for one animal. In Fig. 2, the data show graphically the carotid

pressure (upper curve) and the peripheral pressure (lower curve) after the animal received 1.1 c.c. pitressin two minutes previous to the first reading.

The striking similarity in form of the two curves would seem to indicate the efficiency of the apparatus in measuring peripheral arterial pressure, as well as to show that under these conditions the peripheral arterial pressure so obtained follows the same fluctuations shown by the carotid pressure although of lower amplitude.

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# COMPARISON OF PROCEDURES FOR STAINING TUBERCLE BACILLI IN FLUORESCENCE MICROSCOPY\*

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IN THE detection of tubercle bacilli, uniformly better results have been reported with fluorescence than with the Ziehl-Neelsen stain, the figures ranging from 6 per cent to 40 per cent more positive tests for the former method. The advantages given for fluorescence microscopy are that it stains more bacteria; it is a more rapid method of determination; it is less tiresome for the laboratory technician; and it offers better contrast of bacilli in the field of vision.

Following the early reports of Hagemann<sup>2, 3</sup> in 1937 and 1938, there developed widespread interest in this procedure. Because some investigators have altered the staining technique in some respects, a comparative study of these procedures was felt to be desirable. An experiment was therefore conducted to test the value of various decolorizers and counterstains used in fluorescence microscopy.

## APPARATUS

*Light Source.*—A simple 100 watt mercury vapor lamp placed in a lamp housing and used in conjunction with a special blue glass filter was employed as the source of ultraviolet light radiation.

*Microscope.*—An ordinary monocular microscope was used with a 4 mm. objective N.A. 0.85 together with a 10x ocular. A preliminary comparison of 4 mm. objectives N.A. 0.85 and N.A. 0.66 revealed the former as having a brighter field of vision, although less contrast between the organisms and their background was apparent. The latter type of objective appears to reduce the transmission of light about 60 per cent according to Richards et al.<sup>6</sup> An aluminized mirror was placed over the mirror of the microscope to avoid loss of ultraviolet light rays, and a yellow filter was inserted into the eyepiece.

## PROCEDURE

*Smears.*—Slides of equal thickness were prepared with smears made from a uniform saline suspension of tubercle bacilli. All slides were examined within twenty-four hours after staining, since fluorescence begins to be reduced thereafter.

*Dye.*—Auramine in a 0.1 per cent aqueous solution with the addition of 5 per cent phenol was used. All slides were steamed for five minutes and then washed in tap water.

*Decolorizers.*—Five different decolorizing solutions were used in several strengths and for the time periods as follows:

\*From the Laboratory Dept., Los Angeles Sanatorium, Duarte, Calif.  
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<i>Decolorizer</i>	<i>Time Used</i>
3 per cent concentrated hydrochloric acid in 95 per cent alcohol	Until preparation colorless (ab. 5 min.)
3 per cent concentrated hydrochloric acid in 70 per cent alcohol	Until preparation colorless (ab. 5 min.)
0.5 per cent concentrated H.C.L. and 0.5 Gm. NaCl in 70 per cent alcohol	3 min. (1 min. pour off and renew for 2 min.)
5 per cent concentrated nitric acid in 70 per cent alcohol	30 seconds
25 per cent concentrated sulphuric acid in 70 per cent alcohol	30 seconds

#### *Further Staining.—*

*Potassium Permanganate Solution.*—A series of slides was stained with and without the use of potassium permanganate solution in order to demonstrate its importance in eliminating side fluorescence. A 1:1000 aqueous solution was prepared, into which slides were dipped for one second following decolorization and washing in tap water.

*Methylene Blue Solution.*—Other slides were counterstained with methylene blue solution and compared with smears untreated with this dye, so that its value in offering better contrast between the bacilli and their background could be studied. Loeffler's alkaline methylene blue solution diluted 1:10 was used for three seconds following decolorization and in some cases treatment with potassium permanganate solution. The slides were then washed in running water and dried.

There were a total of twenty fluorescence-stained slides in this study. The presence of tubercle bacilli on each of the slides was verified by a control smear stained by the Ziehl-Neelsen method. Each fluorescence-stained slide was examined for best visibility of tubercle bacilli as compared with other slides in the series. The intensity of light radiation offered by the organisms as they appeared to the eye of the investigator was the deciding factor in establishing the degree of luminescence on microscopic examination.

#### RESULTS

A slide decolorized with a solution of 25 per cent concentrated sulphuric acid in 70 per cent alcohol followed by staining with a 1:1000 aqueous solution of potassium permanganate offered the best visibility. Another slide decolorized with a solution consisting of 3 per cent concentrated hydrochloric acid in 70 per cent alcohol and counterstained with potassium permanganate solution and the methylene blue solution presented a degree of visibility which proved second best. A slide decolorized with a solution of 0.5 c.c. concentrated hydrochloric acid and 0.5 Gm. sodium chloride in 70 per cent alcohol and counterstained with methylene blue solution gave the third best results.

In studying those slides which were not stained following decolorization, the investigator was troubled somewhat by side fluorescence of material such as crystals, tissue cells, fibers, secretions and like particles. However, it was generally observed that somewhat fewer organisms were present on those slides which were counterstained with methylene blue solution.

## DISCUSSION

There is no doubt that an improved source of light which would allow for binocular vision would greatly improve detection of the organisms. Until this is found we must necessarily restrict our improvements to bettering the staining technique.

The dye, auramine O, appears to be the best in use at present. A comparison of this coloring matter with other fluorescent dyes such as fluorescein, rhodamine, tartrazine, chrysoidine and Victoria green showed that none of these could equal auramine in degree of fluorescence when exposed to ultraviolet rays. Hagemann<sup>3</sup> cold-stained his slides at room temperature as did Richards et al., but we preferred the steam-staining period of five minutes as used by Hermann<sup>4</sup> and by Bogen.<sup>1</sup> We felt better assured of thorough fastness of the stain and a lesser effect on them by strong decolorizing solutions which were subsequently used.

In decolorization of his stained slides Hermann used a solution of 3 per cent concentrated hydrochloric acid in 70 per cent alcohol for a period of fifteen to twenty seconds. He then made the observation that if this solution was used for a period longer than three minutes, some of the tubercle bacilli also became decolorized. Thompson,<sup>7</sup> using a decolorizing solution of 1 per cent concentrated hydrochloric acid and 0.5 Gm. sodium chloride in 70 per cent alcohol, treated his stained slides for a period of twenty minutes (ten minutes, pour off, and renew ten minutes). He preferred this stronger acid solution and a longer period of decolorization than that used by Richards from whom Thompson's technique was taken. From the results of these observations and those of this experiment, the stronger decolorizing agents appear to improve the fluorescence of the organisms and prevent interfering side fluorescence. Nevertheless, as stated above, care must be used in accurately timing the decolorizing period to avoid bleaching of the tubercle bacilli.

Fewer organisms are seen with the use of a dye counterstain such as methylene blue. This has been noted also by other investigators and is important on slides with scanty bacilli. Therefore, the use of potassium permanganate solution following decolorization can be sufficient in overcoming interfering fluorescence.

According to Oscarsson,<sup>5</sup> "overdiagnosis" of the slide must be cautioned against, especially in cases with few bacilli present on the slide. This could result from fluorescence of near-by material resembling the morphology of acid-fast bacilli. We found this to be true in our early studies with 100 comparative slides. There was a tendency to interpret a very high percentage of positive results although these subsequently proved to be unfounded in great part when checked by standard control procedures. We are still unable to feel complete reliance in this procedure as an accepted method of examination. Assurance in the diagnosis of tubercle bacilli by fluorescence microscopy is not easily acquired.

## SUMMARY

Lacking improvement in the source of ultraviolet light and in the luminescing dye used in fluorescence microscopy, changes can be suggested in the



use of various types of decolorizing and counterstaining solutions for the detection of tubercle bacilli.

The application of stronger decolorizing agents than those commonly employed appears to be advantageous.

The additional staining of the slides with potassium permanganate solution improves the visibility of the organisms.

Further counterstaining with methylene blue solution seems to be an unnecessary step and may only reduce the number of organisms visible in the field.

Confidence in the use of this procedure is not easily gained.

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## CHEMICAL

### THE MICRODETERMINATION OF AMINO ACID NITROGEN IN BLOOD WITH THE SPECTROPHOTOMETER AND WITH THE OPTICAL COLORIMETER\*

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RECENTLY a modification of Folin's<sup>1</sup> method for the determination of amino acids in the blood using the optical colorimeter was published by Sahyun.<sup>2</sup> He used Danielson's<sup>3</sup> mixed amino acid standard consisting of glycine and glutamic acid. The major change from the Folin method was that the amino acid solution (or the blood filtrate) was heated with the dye in a boiling water bath for three minutes instead of being left in the dark overnight. This materially accelerated the determination of amino acids. Both a macromethod and a micromethod were proposed, but it was found that the color produced in the micromethod was too light for accurate optical colorimetric or spectrophotometric determination. The present study was undertaken in an effort to increase the intensity of the color difference, and hence the accuracy of the method, and to adapt it to the spectrophotometer.

#### EXPERIMENTAL

##### Reagents.—

Sodium Hydroxide, 0.05 N solution.

Sodium Borate, 2 per cent solution.

Phenolphthalein, 0.25 per cent solution.

Sodium- $\beta$ -naphthoquinone-4-sulfonate, 0.5 per cent solution prepared freshly before using. Do not use a solution that has stood for more than 15 or 20 minutes.

Special acetic acid-acetate reagent, 100 c.c. of 50 per cent acetic acid diluted with an equal volume of 5 per cent sodium acetate.

Sodium thiosulfate, 4 per cent solution of  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ .

Stock standard amino acid solution, containing 0.1 mg. of alpha amino nitrogen per c.c. Samples of glycine and proline are dried to constant weight over sulfuric acid in vacuum. Accurately weigh 187.8 mg. of proline and 412.8 mg. of glycine, and dissolve in 0.07 N HCl containing 0.2 per cent sodium benzoate as a preservative. The

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resulting solution is made up to 1 liter in a volumetric flask, using 0.07 N HCl—0.2 per cent sodium benzoate as a diluting fluid.

Working standard amino acid solution, containing 0.004 mg. alpha amino nitrogen per c.c. Prepared by accurately introducing 2 c.c. of the stock standard into a 50 c.c. volumetric flask and diluting to volume.

Dilute tungstic acid solution, 40 c.c. of 10 per cent sodium tungstate in a liter volumetric flask diluted to about 800 c.c. with water. Add 40 c.c. of  $\frac{3}{4}$  N sulfuric acid and dilute to volume. This solution will not keep indefinitely; and whenever a white precipitate of tungstic acid appears in the bottom of the bottle, a fresh solution should be made up.

*Procedure.*—A Model 11 Coleman Spectrophotometer was used. The optimum wave length for this method on the spectrophotometer was found to be 470 m $\mu$ .

Using a calibrated micropipette, introduce 0.2 c.c. of fingertip or oxalated blood into 6 c.c. of the special dilute tungstic acid solution in a 15 c.c. centrifuge tube. Mix well, let stand for about five minutes, and centrifuge. Into a graduated test tube, introduce 5 c.c. of the filtrate and simultaneously measure 5 c.c. of distilled water into another graduated test tube for the blank. Add to each 1 drop of phenolphthalein and neutralize to a definite pink with 0.05 N sodium hydroxide. If on standing a short time the color gradually fades, do not add more, as the color will return when the borate is added. Add 1 c.c. of 2 per cent sodium borate; the color should be of a uniform pink shade in all of the tubes. Then add 0.4 c.c. of freshly prepared sodium- $\beta$ -naphthoquinone-4-sulfonate and mix well by shaking. Immerse in a boiling water bath for three minutes, cool under running water. Add 1 c.c. of acetic acid-acetate reagent followed by 1 c.c. of 4 per cent sodium thiosulfate. Dilute to the 10 c.c. mark, mix by shaking (do not invert unless the tube has a ground glass stopper), and compare colors.

*Standardization of the Spectrophotometer.*—The 0.004 mg. amino acid standard was used to standardize the spectrophotometer by taking the following portions:

- A blank consisting of 5 c.c. of distilled water
- 1 c.c. of standard, corresponding to 2.48 mg. per 100 c.c.
- 2 c.c. of standard, corresponding to 4.96 mg. per 100 c.c.
- 3 c.c. of standard, corresponding to 7.44 mg. per 100 c.c.
- 4 c.c. of standard, corresponding to 10.02 mg. per 100 c.c.
- 5 c.c. of standard, corresponding to 12.40 mg. per 100 c.c.

All samples were diluted to 5 c.c. with distilled water and treated the same as the blood filtrates. The per cent absorption was graphed against the mg. per 100 c.c. concentration in the blood on semilogarithmic paper. Then for all determinations, a blank was used instead of a standard, and the graph was consulted for the concentration.

*Optical Colorimeter.*—When this method was tried on the optical colorimeter, it was found to give results which were 0.2 to 0.5 mg. per 100 c.c. less than on the spectrophotometer. The 3, 4, and 5 c.c. standards, as described under standardization, were used with the standard set at 20 mm.

#### DISCUSSION

In adapting the macromethod to the spectrophotometer, it was found that the only modification necessary was to reduce the amount of 0.25 per cent solution of sodium- $\beta$ -naphthoquinone-4-sulfonate from 2 c.c. to 1 c.c. for each sample. The standardization of the instrument for the macromethod was carried out using portions corresponding to 3.33, 6.67, 10.0, 11.7, and 13.3 mg. per 100 c.c. in blood of either the macrostandard of glycine-glutamic acid as in Sahyun's original macromethod<sup>2</sup> or a macrostandard made from a stock proline-glycine standard.

In the micromethod the principal changes made consisted of a reduction in the amount of dye used, an increase in the concentration with a decrease in its volume of the precipitating reagent, and the substitution of a proline-glycine standard for the glycine-glutamic acid standard.

Recovery experiments were made by adding known amounts of glycine-glutamic acid standard in three different concentrations to blood filtrates of both the micromethod and the macromethod. The average deviation from the theoretical recovery of added glycine-glutamic acid standard in nine experiments was -0.33 mg. per 100 c.c. for the micromethod and -0.14 mg. per 100 c.c. for the macromethod.

A series of 24 oxalated bloods run to test the accuracy of the micromethod as compared to the macromethod showed an average difference of +0.054 mg. per 100 c.c. In most cases the variation amounted to 0 to 0.3 mg. per 100 c.c., and in no case was it greater than 0.7 mg. per 100 c.c. The amino acid nitrogen levels (fasting) ranged from 6.6 mg. per 100 c.c. to 11.4 mg. per 100 c.c., with an average of about 8.

Glutamic acid, glycine, alanine, valine, leucine, phenylalanine, tyrosine, proline, histidine, cystine, arginine, and tryptophan were made up so that 1 c.c. of solution would contain 0.1 mg. of alpha amino nitrogen. From these solutions, mixtures were made up which simulated as nearly as possible the amino acid compositions of casein, lactalbumin, and globin as reported in the literature,<sup>4</sup> because it was felt that these would simulate the amino acid composition of the blood. Proline, in spite of the fact that it has no primary alpha amino group, reacts with the dye and so was added in correct concentration to the mixtures. Micro-Kjeldahl analyses were made of all of the amino acid solutions and were found to agree closely with the theoretical.

Because it was found that proline did react with the dye, pseudoprotein mixtures were studied with and without proline present. It was found that proline had an effect greater than its nitrogen concentration as compared to glycine and glutamic acid. This effect seemed to be enhanced by the presence of arginine and presumably by other basic amino acids.

In Fig. 1 the micromethod calibration curves on the spectrophotometer at wave length 470 m $\mu$  are shown for the two representative pseudoprotein

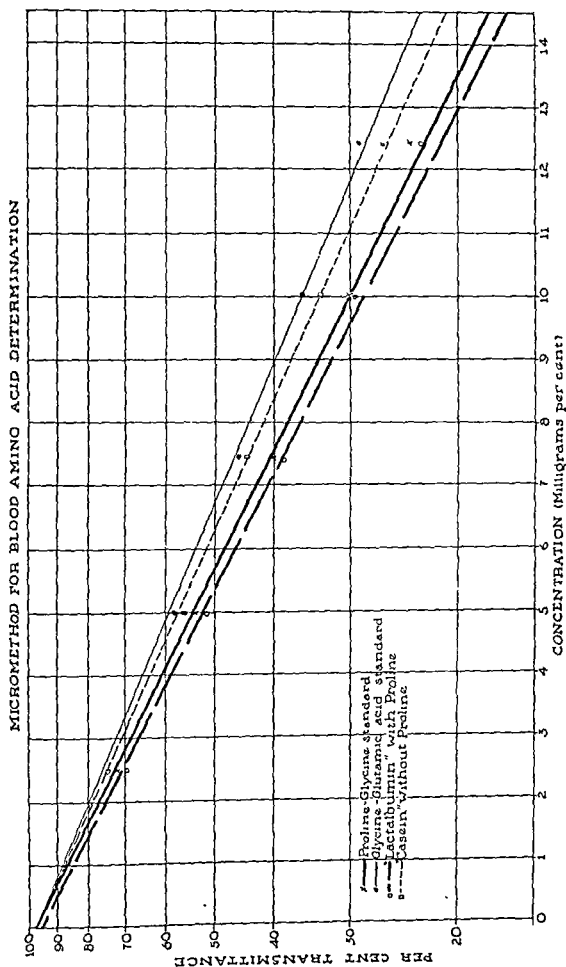


FIG. 1.—Calibration curves for amino acid micromethod using different amino acid mixtures.

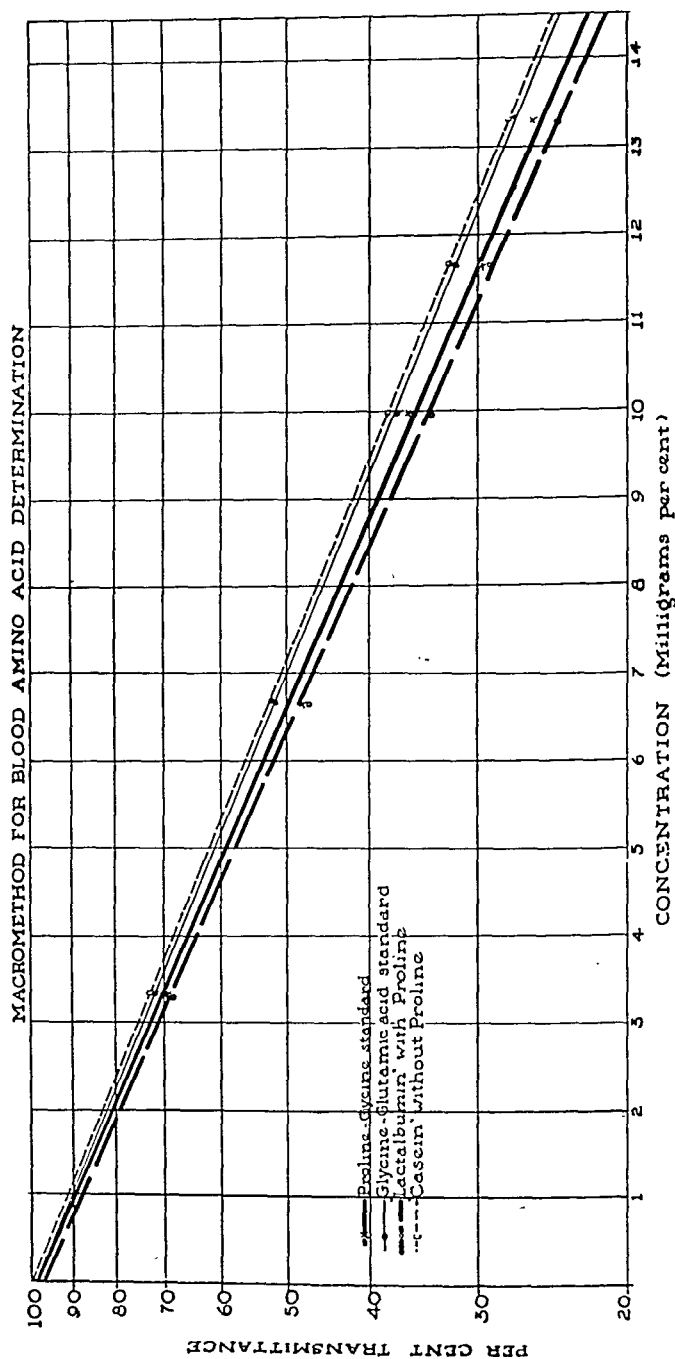


Fig. 2.—Calibration curves for amino acid macromethod using different amino acid mixtures.

mixtures giving respectively the least and the greatest absorption. For comparison the curves for the usual glycine-glutamic acid standard and for the new proline-glycine standard are also pictured. In the proline-glycine standard, 2 parts of the alpha amino nitrogen are due to proline and 7 parts to glycine. Fig. 2 shows similar comparative curves for the macromethod. It can be seen that the proposed proline-glycine standard is more nearly a median between the pseudoprotein extremes in both the micromethod and the macromethod than is the glycine-glutamic acid standard.

#### SUMMARY

A new micromethod for the determination of amino acid nitrogen in the blood, based on the Sahyun modification of Folin's method is described. Various factors which increase the accuracy over the previous micromethod are taken into consideration. The method is adaptable both to the spectrophotometer and to the optical colorimeter.

The author wishes to acknowledge her gratitude to Dr. Melville Sahyun of Frederick Stearns and Company for his helpful suggestions and to Dr. Hans Hecht of the William J. Seymour Hospital for technical assistance in obtaining the blood samples.

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# A NOTE ON A SIMPLIFIED AND RAPID TEST FOR PROTEINURIA

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WITH THE TECHNICAL ASSISTANCE OF CHARLES D. BOYD, U. S. ARMY

**I**N THIS laboratory large numbers of routine urinalyses are performed daily. A test for proteinuria which would involve a minimum of equipment, a simple technique, and accurate results was sought.

The procedure developed represents a qualitative ring test for detection of proteinuria which, by observance of the degree of density of the ring, is roughly quantitative.

The reagent, developed by one of us (C. D. B.), consists of a mixture of equal parts of a 5 per cent aqueous solution of trichloroacetic acid and absolute methyl alcohol. This reagent, in well-stoppered dark bottles, may be kept indefinitely without evident deterioration. The trichloroacetic acid precipitates the urinary protein, and methyl alcohol makes the resulting solution light enough to overlay the urine.

The test is performed as follows: From 5 to 10 c.c. of urine are centrifuged in a centrifuge tube, or ordinary test tube, and this is overlaid with the reagent by dropping four to eight drops from a dropper bottle. The presence of an abnormal amount of albumin is indicated by the prompt formation of a white ring at the junction of the urine and reagent. The most consistent results are obtained when the test is read between thirty to sixty seconds. Following the reading of the test, the urine (and reagent) may be poured out and the microscopic examination of the urine carried out on the sediment.

The advantages of this technique over those in general usage are most evident in a laboratory where a large volume of work is done such as in military hospital laboratories. The procedure is time saving in that no complicated techniques are involved; one centrifugation and one set of test tubes serve for both the protein test and microscopic study. The simplicity of performance and the distinctness of positive tests make it a dependable test for both student and trained technicians.

Trichloroacetic acid is recognized as an efficient reagent for precipitating protein and has been applied to the study of urine in the form of both contact and turbidity tests. Various studies, however, were carried out by the authors to obtain the optimum strength for this particular technique. Studies on both pathologic urine specimens and specimens prepared by the addition of varying quantities of blood serum indicate that the small quantities of protein normally present in the urine are not detected by this test, and amounts generally conceded to be pathologic show positive tests.

This test has been used in an Army Station Hospital laboratory as the routine method for testing for proteinuria for five months, during which time over 4,000 tests have been made. Most of the positives were checked by other



techniques, and on a considerable percentage, quantitative determinations were done. Only one false positive was obtained, this being due to an excessive amount of mucus which was not thrown down by centrifugation. Two hundred consecutive urines negative for protein by the authors' technique were also negative with Roberts' reagent.

Studies of the microscopic sediment from tubes in which this test has been performed show no alteration in either the formed elements or unorganized chemical constituents. As a matter of fact, studies show that the pH of the urinary sediment is not altered.

#### SUMMARY

A simple, time- and equipment-saving contact test for proteinuria using trichloroacetic acid and methyl alcohol has been described. We believe this test will prove to be of considerable value, especially in military laboratories.

## BOOK NOTICES

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### The Neuromuscular Maturation of the Human Infant\*

THE author has demonstrated, within a little over a hundred pages, what to expect in the development of the newborn infant until it arrives at a state where its activities are under cortical control.

The maturation of the infant shows its development to cover periods of transition from a phase where the body activities are under subcortical control to one in which the activities are under cortical control. Through diagrams and illustrations as well as descriptive text, the various phases the infant passes through from subcortical to cortical control are aptly demonstrated.

In describing the infant's growth the author gives the various phases the infant passes through in learning to roll over, crawl, sit, and walk. Diagrams are presented to chart these phases of development so that one infant's activity can be compared with that of another and normality for an individual can be determined.

The author in concluding states that exercise of a newly developing function is inherent in the process of growth, and if ample opportunity is afforded at the proper time, specific achievements can be advanced beyond the stage normally expected. She also concludes that training of any particular activity before the neural mechanisms have reached a certain state of readiness is futile.

After reading the book, which was found to be very instructive and entertaining, the reader feels that he has a much better insight into the neuromuscular maturation of the infant.

L. E. S.

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### The Autopsy†

THE autopsy is the subject of a special war number of the *Journal of Technical Methods* and the *Bulletin of the International Association of Medical Museums*.

The importance of the autopsy along with special bacteriologic, chemical, and other methods in furthering our knowledge of war wounds, diseases of war, and tropical medicine is emphasized in an introduction by the Conference Group on Pathology of the National Research Council.

There follows a very complete discussion of the autopsy, including autopsy permission, the relation of the coroner, regulations of the Army and Navy, equipment and supplies necessary for the autopsy room, and the technique of the autopsy. The techniques described along with the description of the protocol are those usually to be found in the better institutional departments of pathology. Toxicologic, bacterial, and medicolegal examinations are also discussed.

The presentation should serve as an excellent means of securing standard performance of autopsies in the Army and Navy and should prove of value to many pathologists in maintaining the standard of the autopsy.

J. H. S.

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\*The Neuromuscular Maturation of the Human Infant. By Myrtle B. McGraw. Cloth. 140 pages. Morningside Heights, Columbia University Press, New York, 1943.

†The Autopsy. Special war number of the *Journal of Technical Methods* and *Bulletin of the International Association of Medical Museums*, No. XXIII, February, 1943. Cloth, 85 pages. Waverly Press, Inc., Baltimore, Md., 1943.

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## CLINICAL AND EXPERIMENTAL

### STUDIES ON A POLYSACCHARIDE FROM THE TUBERCLE BACILLUS\*

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REFERENCES to the role played by polysaccharide substances in pathologic and nonpathologic processes are numerous and date back some 40 years, when Emerling,<sup>1</sup> and Schardinger,<sup>2</sup> reported the isolation of a nonnitrogenous polysaccharide from *Bacillus luctis aerogenes*. Our more recent knowledge of the chemical and immunologic specificity of polysaccharide materials, especially from the pneumococcus and tubercle bacillus, has been derived in a large measure from the work of Landsteiner, Heidelberger, Avery, Goebel, Sabin, and their associates. Other workers have contributed information concerning this subject.

In view of the above work and since no definite role has been assigned to the polysaccharide fraction in the pathogenesis of tuberculosis, it seemed worth while to attempt the following type of experiment.

#### MATERIAL AND METHODS

The fraction was isolated from the aqueous extract (2000 c.c.) of approximately 4 kilograms of moist germ mass which had been grown on Dorset's synthetic medium. The bacillary mass was mechanically agitated in its aqueous liquor for one month previous to the process of extraction. The fraction when ready for clinical testing was free from organic nitrogen, as was determined by the biuret, ninhydrin, and the sodium fusion (Lassaigne) tests. The material gave a strong Molisch reaction, indicative of carbohydrate substances; it does not reduce before hydrolysis but reduces alkaline copper reagents after hydrolysis in 1 N HCl. Tests for combined pentoses were positive. The optical rotation (observed) was 58 D. The naphthoresorcinol test for glycuronic acid was

\*From the Hospital Department, Sanatorium Division of the Boston City Hospital.  
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negative. The fraction was completely soluble in water at ordinary room temperature. Further chemical investigation of the carbohydrate material is now under way.

#### TOXICITY

The tests for toxicity were carried out in tuberculous and nontuberculous guinea pigs. Temperatures were taken in each case before any injections were made. Twenty milligrams in 1 c.c. of normal saline solution given intraperitoneally was found to be the lethal dose for animals with generalized tuberculosis. Death followed within five to eight hours after administration of the lethal dose. Normal saline solutions were injected into control animals. The carbohydrate material produced a rise of 3 to 5 degrees in the tuberculous animals and from 1 to 2 degrees in nontuberculous animals. The temperatures in the infected animals continued to fall to 95° F. at the time of death. In the nontuberculous animals the temperatures did not fall to more than 2 degrees below the temperatures taken before injection; none of the nontuberculous animals succumbed to the dosage used. No rise in temperature was observed in the control animals given 1 c.c. of the saline solution alone. It was also noted during post-mortem examination of the tuberculous animals that those with far-advanced disease succumbed sooner than those with less or minimal amounts. Death in the tuberculous animal followed three to eight hours after injection, depending upon the extent of disease present.

#### CLINICAL

After repeated animal experiments of the above character, one of the writers (G. V. K.) and 14 members of the hospital staff, received intracutaneous injections of 0.01 mg. in 0.1 c.c. amounts of the material made up in normal saline solution. Saline controls were used by similar route in the opposite arm, the saline being the same solution used for dilution of the carbohydrate material. Five of the latter group were arrested cases of pulmonary tuberculosis; the remainder had no history of active disease. The results of the skin tests in this group are listed in Table I.

It has been noted during injections of this carbohydrate fraction that when the material is administered subcutaneously, no reaction is seen in the skin whether in tubercular or nontubercular individuals. It has also been observed that in each instance when a positive skin reaction is elicited there occurs a concomitant change in the differential blood picture, i.e., a rise in lymphocytes with a fall in the monocytes and a shift to the right in the Schilling index. The latter change has, in the majority of cases, been correlated with the degree of skin reactivity. For example, the differential blood count of an individual (tubercular or nontubercular) with a  $\pm$  or a  $+$  skin reaction (immediate reaction) has never reflected any noteworthy change following intracutaneous injections of this polysaccharide fraction. As a matter of note, the immediate type of reaction, although in many instances has been correlated with the clinical improvement of the patient, has nevertheless not been constant enough to permit any definite conclusions at this time regarding its clinical significance.

An unusually severe reaction to the intracutaneous injection was seen in subject F. B. An area of erythema appeared within 2 hours following the injection; this gradually became intensely indurated and at its peak measured 8 cm. in diameter. One of the axillary nodes became enlarged and tender. The reaction did not subside until 48 hours later and it was not accompanied by any febrile reaction or local necrosis. This individual gave no past history of active disease.

In each case, total white, red, and differential blood counts were made one hour before injections were given, and counts were again made 8 hours following the injection. In the absence of any skin reactivity whatsoever, no changes in the blood picture have ever been observed. The time of appearance of a positive test in a case of minimal disease, employing the usual dosage of .01 mg., is approximately 3 hours from the time of injection and is completely faded at the end of 20 hours. In the instance of far-advanced disease, however, the reaction is delayed, and if it appears at all, it will not do so until 10 to 12 hours following injection. In many far-advanced cases of this type the increment of dosage has had to be increased (i.e., 0.01 mg. to 0.1 mg.) in order to elicit a local reaction, which then might appear in 5 to 10 hours or longer. In such cases, the reaction has been seen to persist for from 72 to 124 hours.

TABLE I  
SKIN REACTION IN ARRESTED AND NONARRESTED SUBJECTS

SUBJECT	SEX	AGE	AMOUNT	ROUTE	LOCAL REACTION	
					IMMEDIATE	DELAYED
A. C. xx	M	36	0.01 mg.	I.V.	+	++ ( 4 hrs.)
A. E. xx	M	33	0.01 mg.	I.V.	+	++ ( 3 hrs.)
W. R. xx	M	31	0.01 mg.	I.V.	+	++ ( 7 hrs.)
A. M. xx	M	44	0.01 mg.	I.V.	+	++ ( 4 hrs.)
W. K. xx	M	45	0.01 mg.	I.V.	+	++ ( 3 hrs.)
M. G.	F	23	0.01 mg.	I.V.	±	0
C. W.	M	30	0.01 mg.	I.V.	0	++ ( 2 hrs.)
P. C.	M	30	0.01 mg.	I.V.	±	0
G. M.	M	28	0.01 mg.	I.V.	+	0
E. R.	F	23	0.01 mg.	I.V.	0	++ ( 6 hrs.)
G. M.	F	29	0.01 mg.	I.V.	0	0
E. J.	F	31	0.01 mg.	I.V.	±	++ ( 4 hrs )
M. B.	M	33	0.01 mg.	I.V.	0	0
F. B.	M	34	0.01 mg.	I.V.	0	++ (48 hrs.)
G. V. K.	M	38	0.01 mg.	I.V.	+	0 (15 hrs)

+ Wheal with erythema and no induration 2 cm. or more; 5' to 15'.

++ Wheal with erythema and induration 2 cm. or more, 2 to 10 hours' duration.

xx Arrested cases of at least 2 years' standing.

± Erythema less than one cm., with no induration

In Table I there is noted a delayed reaction in three individuals with no past history of disease. We have not as yet been able to establish any correlation in such instances between the individual's past history or present status and the skin reaction. The discussion of the latter type of reaction will be considered under the serologic heading. On repetition of the same dose, the size and intensity of the skin reaction is seen to fade until a local reaction is no longer elicited. The phenomenon is dependent upon the intensity and duration of the initial reaction. As mentioned above, a local reaction is again elicited when the increment of dosage is increased.

For the sake of brevity, and because more detailed tables and data are presently being prepared, we wish to present a group of 67 patients with minimal and far-advanced disease in whom injections of the polysaccharides were given without hematologic or serologic study, in an endeavor to show some correlation between the local reaction and the extent of disease present in the individual case. In Table II is a summary of the patients thus studied:

TABLE II  
LOCAL INTRACUTANEOUS REACTIONS IN MINIMAL AND FAR-ADVANCED DISEASE

NO. OF PATIENTS	STAGE OF DISEASE	RANGE OF DOSE	AV. TIME OF APPEARANCE OF LOCAL REACTION	AV. TIME OF DURATION OF LOCAL REACTION
22	Minimal	0.001 mg. to 0.01 mg.	38 hrs.	19.7 hrs.
45	Far Advanced	0.01 mg. to 0.3 mg.	10.8 hrs.	52.4 hrs.

Our observations in this group justify us in saying that (a) the time of appearance, (b) the intensity and size of the local reaction, and (c) the duration of the local reaction are to be definitely correlated with the extent of involvement in each individual case. This observation suggests some reciprocal relationship to the toxicity tests performed earlier in guinea pigs, when it was noted that animals with far-advanced disease succumbed sooner to the dosage used than did those with a minimal amount. A similar group of patients are now being studied with accompanying hematologic and serologic studies.

#### SEROLOGIC

During the course of the above injections, attempts were made to demonstrate any antibody response which might be elicited by injections of the carbohydrate substance. For this purpose, human subjects were divided into three groups: (1) tubercular patients who had received 12 weekly injections in doses ranging from 0.001 mg. to 0.3 mg., (2) tubercular patients who had received no injections at all, and (3) nontubercular individuals who likewise had received no injections of the carbohydrate substance. The sera of all three groups were tested for the presence of antibody before any injections were made. The technique used in determining the presence of antibodies is as follows: 5 c.c. of blood were drawn from the antecubital vein and placed in a glass tube and allowed to clot. The clot was later separated and the supernatant blood centrifuged at low speed. The sera were then heated at 55° C. for one hour. Dilutions in the sera of 1 to 10 were made in all instances.

The method of titration used was that of the simple ring test, in which the serum was overlayed with an 0.01 mg. per c.c. concentration of the carbohydrate fraction in saline. An equal number of saline-carbohydrate controls were also used. The observations made on the sera of the above subjects are given in Table III.

In Table III there are noted tubercular and nontubercular individuals as well as a convalescent patient having pleurisy with effusion, whose sera contained homologous antibodies for the carbohydrate fraction previous to any injection of

the latter. This phenomenon is altogether possible in tubercular individuals, since the carbohydrate fraction is a component part of the bacillary body. The instance of the convalescent pleurisy with effusion might also be explained on this basis. It becomes, however, more difficult to explain the presence of demonstrable antibodies in the serum of nontubercular subjects who have no past history of active disease, with only the possibility of an earlier childhood type of infection. The persistence of circulating antibodies for an extended period of time is possible since one cannot easily determine the status quo of a primary complex. Recent exposure to respiratory and other types of infection must also be considered. Since the above subjects are members of the sanatorium staff and have been in intimate contact with tuberculous patients for a period of time ranging from two to seven years, the presence of circulating antibodies in these individuals is understandable on this basis.

TABLE III

SERUM REACTIONS OF A VARIETY OF SUBJECTS TO THE CARBOHYDRATE FRACTION

SUBJECT		CLINICAL STATUS*	NUMBER INJECTIONS	AMOUNT	SERUM BEFORE INJECTION†	SERUM AFTER INJECTION†	SALINE CONTROLS
Tubercular Non- treated	H. F.	Min.	0		0	0	0
	R. K.	F. A.	0		0	0	0
	M. F.	Min.	0		×	0	0
	W. C.	F. A.	0		0	0	0
	H. L.	Min.	0		0	0	0
Tubercular Treated	M. K.	F. A.	10	0.001 mg.-0.3 mg.	0	×	0
	H. S.	F. A.	10	0.001 mg.-0.3 mg.	0	×	0
	R. P.	F. A.	10	0.001 mg.-0.3 mg.	0	×	0
	M. C.	Min.	10	0.001 mg.-0.1 mg.	0	×	0
	W. D.	Min.	10	0.001 mg.-0.1 mg.	0	×	0
Non- tubercular Nontreated	P. C.	Nontb.	0		0	0	0
	E. J.	Nontb.	0		0	0	0
	A. M.	Nontb.	0		×	0	0
	A. C.	Nontb.	0		×	0	0
	C. W.	Nontb.	0	0	0	0	0
Con- valescent patient Pleurisy with effusion	R. S.	Nontb.	0	0	×	0	0

\*Min. = minimal disease

F.A. = far-advanced disease

†× = slight ring at junction of fluids

×× = medium ring at junction of fluids

××× = heavy ring at junction of fluids

The type of antibodies elicited by intracutaneous injections of this material, from determinations made thus far, has been precipitin in nature only. The fact that the polysaccharide fraction used in the present experiment has repeatedly stimulated the homologous type of antibody might lead one to question how a hapten-like material may apparently act as a complete antigen. Investigations on polysaccharide substances have shown them to be haptens because of their failure to stimulate antibody formation, the latter being a distinction set forth by Landsteiner. More recent observations, however, have been recorded, and they suggest that certain of the more complex haptens may act as antigens under definite conditions. Thus, the polysaccharide fractions that have been separated from various types of pneumococci, when tested by

ordinary methods of inoculation into laboratory animals, have no antigenic action; on the other hand, when these haptens are injected into the skin of human subjects in minute doses, specific antibodies are formed (Francis and Tillett,<sup>3</sup> Finland and Sutcliffe.<sup>4</sup> The animal and human experiments with the fraction used in this instance corroborates the observations of the above-mentioned workers; namely, that no antibody response has been demonstrated by any other route than the intracutaneous one.

Since the question of protein-nitrogen-contaminating polysaccharide fractions is constantly being raised, we felt it worth while to skin-test a group of 17 nontubercular individuals with the purified protein derivative (P.P.D.) and our polysaccharide fraction. The age range in this group was from 6 months to 49 years. The entire group was first tested with P.P.D. The first strength dose of 0.0002 mg. was administered on the volar aspect of the forearm. Two weeks after all reactions to the protein had subsided, the group was tested with 0.01 mg. of the polysaccharide fraction. The results of these tests are summarized in Table IV.

These cases are being presented for what they are worth; we feel, however, that the results suggest some difference in the nature of the two fractions used. The results could be discussed at length from several points of view; we are, however, reserving this discussion until a similar and larger group which is now being studied in greater detail is completed.

To further investigate the nature of these antibodies, protection experiments against what had previously been found to be the lethal dose in tubercular guinea pigs were carried out with the serum of the three types of subjects mentioned in Table V.

For this purpose tuberculous animals were used. All of the latter had been inoculated four weeks previously with 0.01 mg. strain H37 in the right groin and reacted with two and three plus skin reactions to 1 O. T. intracutaneously. The sera of all the patients used in this experiment were tested for the presence of antibodies previous to injection in the animals. In all the sera of the patients used, there were no demonstrable antibodies found, with the exception of the treated group. Since no quantitative methods were used in the titration of the above sera, we are unable to state definite titers of antibodies occurring in the sera used. One animal in the treated group succumbed to the lethal dose. This animal when sacrificed was found to have an extremely far-advanced disease. Likewise, when using the sera of the non-treated tubercular patient, one animal survived the lethal dose. This particular patient had previously shown definite clinical improvement.

This is in keeping with some earlier observations which were made with the serum of patients who had shown general clinical improvement with marked clearing in their x-ray report. The majority of these patients' sera reacted with the polysaccharide material in the dilutions and technique mentioned above. Since the carbohydrate substance with which we are here concerned is still in the process of chemical study, and since the serologic techniques here employed are crude, one cannot be dogmatic in the interpretation of the above results. For instance, the fact that the precipitin type of antibody was the



only type demonstrable does not rule out the possibility of other neutralizing agents which are not easily demonstrated in vitro and come under the heading of protective type of antibodies. More definite conclusions on this particular

TABLE IV  
SKIN TESTS IN A NONTUBERCULAR GROUP

P. P. D.			POLYSACCHARIDE FRACTION			
CASE NO.	AGE AND SEX	DOSE AND ROUTE	LOCAL REACTION†	2 WEEKS LAPSING TIME	DOSE AND ROUTE	LOCAL REACTION
1	♂ 6 mos.	0.0002 mg. I. Q.*	+		0.01 mg. I. Q.*	-
2	♀ 6 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
3	♀ 16 yrs.	0.0002 mg. I. Q.	-		0.01 mg. I. Q.	+
4	♂ 17 yrs.	0.0002 mg. I. Q.	-		0.01 mg. I. Q.	+
5	♀ 19 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
6	♂ 20 yrs.	0.0002 mg. I. Q.	-		0.01 mg. I. Q.	+
7	♂ 24 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
8	♀ 25 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
9	♀ 25 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
10	♂ 30 yrs.	0.0002 mg. I. Q.	-		0.01 mg. I. Q.	+
11	♀ 23 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
12	♂ 6 yrs.	0.0002 mg. I. Q.	-		0.01 mg. I. Q.	-
13	♀ 31 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
14	♂ 36 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
15	♀ 49 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
16	♂ 30 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
17	♂ 39 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+

\*Intracutaneously.

†+ = Erythema and induration 2 cm. or more in diameter.

TABLE V

PROTECTION EXPERIMENTS IN TUBERCULAR GUINEA PIGS WITH TUBERCULAR-TREATED, TUBERCULAR-NONTREATED, AND NON-TUBERCULAR SUBJECTS' SERUM

ANIMAL	SERUM	AMOUNT AND ROUTE	TIME OF INJECTION	LETHAL DOSE AND ROUTE	TIME OF INJECTION	RESULTS	TIME
Buff	Treated Tb. +	2 c.c. I.V.*	9 A.M.	20 mg. in 2 c.c. I.P.†	10 A.M.	Killed	48 hrs.
Black	Treated Tb. +	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Killed	48 hrs.
Red and White	Treated Tb. +	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	16 hrs.
White	Non-Treated ++ Tb.	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Killed	48 hrs.
Brindle	Non-Treated ++ Tb.	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	12 hrs.
Brindle and Black	Non-Treated ++ Tb.	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	48 hrs.
Black and White	Non-Tb. +++	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	11 hrs.
Brown and White	Non-Tb. +++	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	15 hrs.
Brown and Buff	Non-Tb. +++	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	20 hrs.

+ Tubercular patients having received 12 weekly injections of the carbohydrate fraction in doses of 0.01 mg. to 0.1 mg.

++ Tubercular patients having received no injections.

+++ Nontubercular individuals.

\*I.V. Intravenous.

†Intraleptural.

phase of the work will be possible only, when present chemical, animal, and human investigations are completed.

As was mentioned previously, there follows after each injection of this material (providing a positive skin reaction, delayed type) a change in the differential blood picture, characterized by a rise in lymphocytes and fall in monocytes with a concomitant shift to the right in the Schilling index. We have purposely not included tables or charts concerning these changes, because a larger group of tubercular and nontubercular subjects, along with animal experimentation, is presently going on in an attempt to establish a definite correlation between the subject's present disease status and (1) the hematologic, (2) the serologic, and (3) the skin reactions following intracutaneous injections of the carbohydrate material. It is nevertheless possible for us to state at this time that in instances where there has been an antibody response to injections of this fraction in tubercular individuals, such a response has always been preceded by a change in the lymphocytic cells as mentioned above. The latter observation is in keeping with the recent report of Ehrlich and Harris.<sup>2</sup> The changes produced in the differential blood picture during the course of injections with this carbohydrate fraction varied greatly in their duration from subject to subject. Some blood changes were seen to persist only during the height of the local reaction; others continued for two to three days after the local reaction had subsided. We have not, as yet, made any correlation on this aspect of the problem other than to associate it with the extent of disease in the individual case.

## DISCUSSION

In reviewing the literature pertaining to the leucocytic blood picture in tuberculosis, there is an unquestionable relationship between the lymphocytic-monocytic ratio and clinical status of the patient. From observations made on this subject, an increase in the number of lymphocytic cells and a corresponding fall in monocytes present a blood picture which in the large majority of instances has been closely related to the clinical improvement of the patient. From our own studies with the polysaccharide fraction we have found the afore-mentioned observations to hold true in almost all of our cases. While we do not have, as yet, any data on hand to explain the mechanism by which this polysaccharide fraction influences the lymphocytic tissue, or to assign any definite role to the lymphocyte in tuberculosis, we wish to quote the recent work of Ehrlich and Harris.<sup>2</sup> Although the antigens used in their work (typhoid vaccine and sheep erythrocytes) are different from the one used in our own experiments, the mechanism of response in both instances is strikingly similar. They write in conclusion: "Antibodies began to appear in the efferent lymph 2 to 4 days after injections of the antigen and reached their highest titre after 6 days. This was preceded by a sharp rise in the output of lymphocytes through the efferent lymph, while in the lymph nodes there was lymphatic hyperplasia after preliminary infiltration of granulocytes and monocytes. The fact that the tissue response accompanying the formation of antibodies was chiefly a lymphocytic one points to the latter as a factor in the formation of antibodies."

In our own studies in human beings we have corroborated the latter observation, since a rise in lymphocytes has always preceded the demonstration of any antibodies. Our technique consists of weekly injections of the dosage found necessary to elicit skin reactivity: i.e., usually from 0.001 mg. to 0.1 mg., dependent upon the extent of disease in the individual patient. The primary response seen in the blood has always been a lymphocytic one. The earliest antibody appearance in any of our cases was after 12 weekly injections of doses ranging from 0.01 mg. to 0.2 mg. Our increment of dosage is guided by the persistence of the skin reaction; that is, the increment is increased only after cessation of skin reactivity to the present dose. Experiments have already been mentioned concerning the antibody response.

At present we have no experimental evidence of the role played in tuberculosis by either the antibody or the lymphocyte stimulated by injections of our polysaccharide fraction. Krause, in his essay "The Evolution of the Tubercle," writes, "In the elementary tubercle of ordinary experiment there exists besides the epithelioid cell, the lymphocyte, which in this location does not change its morphological character." Furthermore, the observations of Maximow<sup>6</sup> and Gerard<sup>7</sup> on tissue culture growths conclude that the lymphocyte is a transitional cell and enters into the mechanism of tissue repair.

While the above observations are interesting and although we have made our own notations along this line, we do not mean to place the entire responsibility of repair in tuberculosis upon the lymphocytic tissue or upon the antibodies which might arise therefrom; we do, however, feel that the work done thus far on lymphocytic tissue by various workers should not be entirely cast aside, until further experimental evidence has been presented.

## CONCLUSIONS

1. A protein-free polysaccharide fraction has been isolated from aqueous extracts of the tubercle bacillus that is innocuous in the amounts used in tuberculous and nontuberculous human beings.

2. Hematologic studies of people receiving intracutaneous injections of this polysaccharide indicate a definite trend in the lymphocytic-monocytic ratio, characterized by an increase in lymphocytes and a decrease in monocytes, with an accompanying shift in the Schilling index to the mature type of neutrophilic cell.

3. Patients having received at least 12 weekly injections of this carbohydrate fraction develop antibodies of an homologous type.

4. The antibodies formed in tubercular patients as a result of the above injections are capable of protecting guinea pigs with generalized tuberculosis from succumbing to lethal doses of the polysaccharide fraction.

5. No noteworthy change in the lymphocytic-monocytic ratio is observed unless there is an accompanying positive skin test of the type mentioned in Table I.

6. The sera of some tubercular patients who have not received injections of the carbohydrate material and who have shown definite clinical improvement will protect guinea pigs with generalized tuberculosis against lethal doses of the carbohydrate fraction.

7. The sera of nontubercular humans thus far observed will not protect animals against the lethal dose of the fraction, unless the individual has elicited a typical local reaction.

8. The appearance and duration of the local reaction is related to the extent of disease.

9. Demonstrable antibodies have always been preceded by a rise in the percentage of lymphocytes following injections of the polysaccharide fraction.

10. The skin reaction produced by the polysaccharide fraction used in the above experiments differs in (1) time of appearance, and (2) duration, from the protein fractions used in routine clinical testing.

11. In no instance during the course of the above injections has there been noted any febrile reactions or local necrosis in the amounts used.

12. A group of nontubercular individuals are presented; they were tested with the protein fraction P.P.D. and the polysaccharide fraction used in the present experiment.

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# CHLOROPHYLL: AN EXPERIMENTAL STUDY OF ITS WATER-SOLUBLE DERIVATIVES\*

## IV. THE EFFECT OF WATER-SOLUBLE CHLOROPHYLL DERIVATIVES AND OTHER AGENTS UPON THE GROWTH OF FIBROBLASTS IN TISSUE CULTURE

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THE problem of wound healing is one which has been intermittently the object of intensive clinical investigation over the years because of its obvious importance not only in ordinary civilian practice but especially in wartime. More recently, laboratory methods have been utilized more and more in the search for a satisfactory answer to the question. Tissue culture technique has been found to lend itself particularly well to the testing of various agents for their growth-stimulating effects. By this procedure standard cultures of cells can be grown under uniform conditions, very much as feeding experiments are made use of in studying and assaying vitamin requirements and content of foods. Similarly, single agents in varying concentrations can be added to the basic culture medium, and an exact quantitative measurement of their effect on cell growth determined.

Because of the almost regular response clinically of longstanding chronic ulcerative lesions to the application of chlorophyll with the formation of healthy granulation tissue and subsequent epithelization, as reported by Boehringer,<sup>1</sup> Buergi,<sup>2</sup> Gruskin,<sup>3</sup> and others, it was decided to study the effect of chlorophyll on the growth of fibroblasts in tissue culture to determine if possible whether this agent, as claimed, actually possessed any growth-stimulating properties. Various control experiments were carried out simultaneously, using other substances currently in use or under investigation in respect to the whole problem of wound healing. The preparation used in these studies consisted of medicinally purified water-soluble derivatives of chlorophyll,† in the main, a saponified metal complex, presumably sodium copper chlorophyllin. This we had found from experience to be more satisfactory than the oil-soluble derivatives with which apparently Boehringer and Buergi worked.

The following agents were employed in this study, all except the "ultrafiltrate" in aqueous solution:

1. Chlorophyll,‡ 0.2 per cent
2. Castilian malva§ infusion made by steeping 10 Gm. of

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†Wherever the term "chlorophyll" is used in these studies, the water-soluble derivatives are meant, the term "chlorophyll" being used solely for the sake of brevity.

‡The aqueous soluble chlorophyll preparation was generously supplied by the Rylan Company of New York, sole appointee of the Lakeland Foundation, Chicago, in accordance with the regulations of the Federal Food, Drug, and Cosmetic Act regarding experimental therapeutic products.

§The Castilian malva was generously supplied by the Upjohn Company of Kalamazoo, Mich., in accordance with the regulations of the Federal Food, Drug, and Cosmetic Act regarding experimental therapeutic products.

powdered leaves in 100 c.c. of distilled water for one hour, filtering and autoclaving.

3. Methionine (Merck) 0.01 per cent
4. Sulfanilamide (Merck) 1 per cent
5. Ascorbic acid 0.01 per cent (Merck)
6. Brewer's yeast extract (B complex) 0.025 per cent (Mead)
7. Simms' "ultrafiltrate"\*

#### PROCEDURE

The technique employed is essentially that successfully used by us for the past ten or twelve years in tissue culture studies.<sup>4</sup> Especially designed thin 0.5 cm. Pyrex glass or aluminum rings with an inside diameter of 4 cm. are mounted sterilely on a cover glass by the use of paraffin. The medium is then added to this chamber, the tissue fragments fixed in position, and the chamber sealed by another sterile cover slip. This entire chamber is then placed within a sterile Petri dish and incubated in the usual manner. By this technique we have an easily accessible means of washing our cultures and replenishing the medium. It furthermore gives us a large, flat surface area for microscopic examination and photography of the culture and permits the use of a relatively large number of individual explants under identical conditions, a matter of considerable importance in the statistical evaluation of such studies as these, in which individual variation in the behavior of colonies is so prone to occur.

The basic medium used has been a Tyrode-plasma-tissue extract combination in the proportions of 4-4-2 parts respectively. The Tyrode solution is the usual standard salt mixture; the plasma has been obtained from chickens and heparinized in the proportion of 1 mg. to each 5 c.c. of plasma; as a matter of convenience maternal mouse spleen has been used for the tissue extract. After much preliminary and comparative study of tissues from various animals as well as human biopsy material as a source of fibroblasts, the embryonic mouse heart was selected as the test tissue. This possessed the advantage of uniformity for comparative purposes, and likewise was almost constantly available from our breeding colony.

The embryonic mouse heart was minced into small fragments 3 to 4 mm. in diameter and thoroughly washed twice in normal salt solution to remove any adherent tissue extract which might act as a growth stimulant. The fragments were planted in the medium, their identity duly recorded on the cover slip, and their measurement taken. Five to ten fragments were thus inoculated into each culture dish. Readings were made routinely each twenty-four hours, and the growth in millimeters from the edge of the original tissue mass was recorded.

Approximately five hundred tissue cultures were grown, averaging fifty to sixty for each set of experiments. Variations in the concentration of the drug or substance under investigation were made in an attempt to see whether there was any optimal concentration in any instance. In general, it may be said that there was very little difference in the rate of cell growth within wide

\*Courtesy of Dr. H. H. Simms, Columbia University, College of Physicians and Surgeons, New York, N. Y.

limits of such concentration. Too great an excess did seem to exert a definite<sup>\*</sup> inhibitory effect. This might best be explained perhaps on a physical rather than a chemical basis.

Certain technical difficulties were encountered from time to time. A small proportion of the cultures (5 per cent) failed to grow at all. These were simply disregarded in the computations, as the number of such nonviable colonies was about the same in the several groups. The chlorophyll exerted a definite and fairly quantitative inhibitory effect upon the plasma-clotting mechanism\* which vitiated one entire set of cultures. It was found for this reason that concentrations of over 0.2 per cent chlorophyll could not be used successfully with this particular technique, but that the typical optimal growth response was obtained with this concentration.

The Castilian malva offered a special problem, as in the initial experiments the great majority of cultures became contaminated with a mold in spite of the fact that the infusion had been filtered through cotton and autoclaved before using. We had to resort to fractional sterilization to overcome this difficulty. With this exception, contamination was negligible, less than 1 per cent, and such cultures were discarded from the final computations.

Every effort has been taken to make these readings as objective and critical as possible, the measurements being checked by two observers for the most part and frequently without prior knowledge as to which agent was being used.

#### DISCUSSION

The results obtained in these experiments are summarized in Table I. It seems possible to draw certain definite conclusions from an analysis of these figures. They likewise provoke much of speculative interest. There seems to be little purpose in discussing in detail the individual experiments from which this report has been assembled. To those who are interested in the "toil, tears, and sweat" which are involved in such tissue culture studies, the original records are all available. To those whose interest lies primarily in the possible practical application of such studies to the clinical problems of wound repair, such individual protocols would have but little significance.

Nevertheless, there are certain aspects of these studies which might be discussed briefly. In the first place, it is well to emphasize the quantitative nature of these studies. Were it not for the relatively great numbers, their interpretation might very justly be open to criticism. In almost every group there have been individual colonies within a single culture which have temperamentally varied greatly in their rate of growth from their fellows, either lagging or stepping up their cell division to an excessive rate. It is hard to explain satisfactorily such behavior. We believe that the laggards probably represent tissues which have been unduly traumatized in handling; and that the more aggressive colonies retain some of the growth-stimulating factors normally present in injured tissues through surface adsorption or inadequate washing in saline before planting. Such an hypothesis is more or less substantiated by actually macerating cells before explantation, with resultant failure of growth

\*As a side issue, this effect upon clotting was studied on several samples of blood, and it was found that clotting could be delayed or actually inhibited by the addition of chlorophyll in concentrations of 1:200 to 1:50 to blood samples as drawn hypodermically for clotting time. This might be further explored as a possible substitute for heparin or dicoumarin therapy in surgery.

on culture or the addition of supplemental tissue extract to a control culture with corresponding accelerated and more profuse growth.

It will be noted from an examination of the table that the substances studied fall into three groups insofar as their initial effect on cell growth in tissue culture is concerned. The growth of cultures to which either vitamin B or C was added did not appreciably differ from the control cultures. The addition of sulfanilamide definitely slowed down the rate of growth, and this effect persisted throughout the period of observation. In the third group we find that all four agents studied gave evidence of having a certain, comparable stimulating effect upon cell growth during the first twenty-four hours. Even in this group, however, the chlorophyll effect stands out as being significantly greater than that of any of the other three substances. It is of particular interest to note that this initial effect is sustained throughout the duration of the experiments in the case of the chlorophyll, whereas it drops off within forty-eight to seventy-two hours in those cultures to which the methionine, Castilian malva, and Simms' ultrafiltrate had been added, and the subsequent growth more or less parallels that of the control cultures. This same stimulating effect of chlorophyll upon fibroblastic proliferation in tissue culture has been observed by Dunham.\*

TABLE I  
AVERAGE GROWTH OF FIBROBLASTS EXPRESSED IN MILLIMETERS

	24 HRS.	48 HRS.	72 HRS.	96 HRS.	120 HRS.
Control	10.8	37.4	67.8	99.3	132.5
Vitamin B	11.3	44.1	70.2	98.7	129.8
Vitamin C	10.6	38.6	59.4	91.3	123.7
(Ascorbic Acid)					
Sulfanilamide	5.3	18.3	31.7	48.8	63.4
Ultrafiltrate	23.2	44.1	69.3	86.4	95.6
Methionine	22.8	23.2	72.5	87.5	130.0
Castilian Malva	22.7	40.5	63.5	85.8	126.4
Chlorophyll	27.4	54.3	88.9	120.4	148.6
(Aqueous Solution)					

These studies have likewise emphasized the necessity of replenishing the supplementary stimulating agent in the medium in order to maintain the rate of growth of the culture. The practical application of this observation to the clinical use of such preparations is self-apparent. The control culture grows at an average rate of approximately 31 mm. per twenty-four hours after an initial lag period of six to eighteen hours. The same picture is noted in those cultures to which vitamin B complex or ascorbic acid is added. There is the same relatively prolonged lag period, followed by a steady, continuous growth apparently uninfluenced by these agents. On the other hand, the sulfanilamide appears to maintain a continuous depressant effect over a period of at least one week, following a single dose added initially to the medium, unless the dilution is extremely high (1:5000). In the latter case no abnormality in the growth curve develops as compared to the control. An almost comparable retardation in the rate of growth occurs at whatever point during the first five days that the sulfa drug is added.

\*Unpublished Data; Dunham, W. B., New York Post-Graduate Hospital, New York, N. Y.



In the case of the Castilian malva, the Simms' ultrafiltrate, and the methionine, the initial stepping up of the growth curve is followed by a retardation in the rate of growth of the culture, and subsequent replenishment of the medium with these agents produces only a slight and temporary additional stimulating effect.

With the addition of as little as 0.05 per cent of chlorophyll to the culture medium at the time of its inoculation, there is a sharp reduction in the lag period. Well-defined growth can be demonstrated regularly within six to eight hours, and not infrequently migration of cells occurs within two to four hours. This acceleration of fibroblastic proliferation tends to reach its peak within thirty-six to forty-eight hours, following which the growth curve begins to flatten out. By replenishing the chlorophyll every forty-eight hours, however, the initial 24-hour lead of nearly 40 per cent greater growth is maintained, with a step-like curve. If, however, the culture is not replenished with chlorophyll by the end of the forty-eight-hour period, then the curve of growth slows down so that by the end of five to ten days it, too, parallels the control culture.

The nature of this biologic response of the fibroblast to the presence of chlorophyll is most obscure. Whether it is the pigment molecule as a whole or some individual component part of it which is the effective agent remains undisclosed. Whether the chlorophyll acts directly or indirectly as a catalyst or through some photosynthetic mechanism is but one of the problems which this study provokes and which await a solution at some time in the future.

These tissue culture studies tend to support the clinical observations of Buergi, Gruskin,\* and others as to the apparent growth-stimulating effect of chlorophyll upon fibroblasts. The clinical implications in regard to its possible therapeutic value in the repair of wounds are obvious. Even in ordinary operative procedures it might well have a place through the elimination or reduction of the usual lag period before repair begins. In chronic indolent lesions such as anemic (varicose) ulcers and other chronic infections, especially those associated with a reduced blood supply, the use of such water-soluble chlorophyll preparations would seem to be thoroughly justified. Again, in the older patient, whose healing processes normally are notoriously retarded, chlorophyll would seem to offer a possible step forward in the treatment of the multitudinous surface lesions with which he seems particularly to be afflicted.

#### SUMMARY

1. The behavior of fibroblasts in tissue culture subjected to the action of various agents including vitamin B complex, vitamin C, sulfanilamide, Castilian malva, methionine, Simms' serum ultrafiltrate, and an aqueous soluble chlorophyll preparation is reported.

2. Vitamin B complex and vitamin C cause no change in the rate of growth of fibroblasts under the conditions of these experiments.

3. Sulfanilamide in concentrations of more than 1:5000 show a definite retarding effect upon such cell growth.

4. Castilian malva, methionine, and Simms' ultrafiltrate cause a prompt but temporary increase in the rate of growth during the first twenty-four to forty-eight hours, followed by a leveling off of the curve to approximate the control.

\*Loc. cit.

5. Chlorophyll in concentrations of 0.05 per cent to 0.5 per cent, when added to comparable tissue cultures of embryonic fibroblasts, causes an almost immediate growth response with elimination of the usual six- to eighteen-hour lag period.

6. This growth-stimulating effect can be maintained by replenishing the medium with chlorophyll every forty-eight hours.

7. A discussion of the possible clinical application of this biologic reaction to the problem of wound healing, both traumatic and thermal, is presented.

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## TRAUMATIC AUTOGRAFT OF SPLENIC TISSUE IN THE BODY WALL<sup>\*</sup>

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THERE have been a few reports in the literature of implants of splenic tissue over peritoneum and omentum as a result of traumatic rupture of the spleen. The usual sequence of events is: traumatic rupture of the spleen, splenectomy, and, often years later, observation of the abdominal cavity at operation or autopsy, at which time multiple small nodules of splenic tissue are found studded over the peritoneal surfaces and omentum. At least 82 such transplants in one patient are mentioned by Shaw and Shafi,<sup>11</sup> about 75 in a case of Hamrick and Bush,<sup>5</sup> and 200 to 300 nodules in a case described by Lee.<sup>7</sup> The small implants vary from microscopic size to as large as 2 cm. in diameter and are usually sessile growths, but may be pedunculated.

Most of the implants which have been described are superficial peritoneal masses, but in the case of Shaw and Shafi, one microscopic implant was noted in the wall of the gall bladder, one in the upper portion of the left ureter (a left nephrectomy appears to have been performed on the patient at the time of the splenectomy), and one was imbedded in the liver. It is presumed that this last nodule was a graft which developed in a laceration of the liver which occurred at the time of the original injury. One macroscopic pleural implant was also noted on the lateral aspect of the centrum of the eighth dorsal vertebra. The authors did not demonstrate a scar in the diaphragm, but believed that there must have been a small tear to account for this implant.

That this condition is not often seen is probably due to a combination of factors. Especially important is the infrequent survival of persons who suffer traumatic rupture of spleen (as mentioned by Hamrick and Bush<sup>5</sup> and the probability that relatively few of those who do survive the injury and splenectomy ever come to subsequent operation or autopsy so that the peritoneal cavity can be examined.

From consideration of experimental work, as well as case reports, it seems probable that splenic implants may be the rule rather than the exception in cases of traumatic rupture of the spleen. Several factors may influence the likelihood of implant, however. It is suggested by Hamrick and Bush that youth may be an important factor. They note that the cases where data are available have suffered the original trauma at an average age of slightly more than nine years.

It is possible that youthfulness of splenic tissue is an important factor in viability of this tissue for autografts, but it should be kept in mind that traumatic, subcutaneous rupture of normal spleen occurs most frequently in childhood,<sup>12</sup> and this may account in part for the greater frequency of the phenomenon

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in children. It is noted in different series that incidence of splenic rupture varies from 30 to 43.8 per cent for the age group 0 to 10 years.<sup>12</sup> In animals and in man, the spleen tends to become somewhat atrophic in old age, and this may have some effect on the probability of implant in older age groups. It has been observed in rabbits that autografts of spleen are, on the whole, more successful in young animals.<sup>8</sup>

Other factors which may influence the likelihood of implantation are by no means clear. In their review, Jarcho and Andersen<sup>6</sup> reported two cases, discussed twelve other cases, and examined certain of the older theories suggested to account for the presence of many splenic nodules over the peritoneum after recovery from traumatic rupture of the spleen. One of the most interesting of the older ideas is the "splenoid" theory, largely accredited to Von Stubenrauch. According to this theory, removal of the spleen causes the formation of nodules of tissue resembling spleen by differentiation of previously undifferentiated cells of the peritoneum. Another theory suggested that the nodules represent splenic anlagen which are stimulated to undergo compensatory hyperplasia as a result of splenectomy.

It should be mentioned that one or more accessory spleens are frequent. It has been suggested that they account for the multiple splenic nodules after splenectomy following traumatic rupture. Accessory spleens, however, occur characteristically in the region of the splenic bed and are never widespread as in the case of traumatic implants. Shaw and Shafi, and others, have pointed out that the distribution of splenic implants over the peritoneum, diaphragm, omentum, and in the pelvis is like that observed in implants seen in intra-abdominal malignancy where scattering of viable tumor tissue is presumed. Also, the fact that implants are observed only in cases where the spleen is removed for traumatic rupture, and never after removal of the intact spleen, is good evidence that the nodules observed are autografts of the ruptured spleen.

At this time there can be no doubt that traumatic rupture of the spleen can result in wide distribution of fragments of splenic tissue which may persist as implants over the peritoneum for many years. It would be reasonable to suppose that these autografts, which resemble normal spleen, might be capable of reacting to disease as does intact spleen. It is the purpose of this paper to describe a case which has two features of special interest. First, it is an example of traumatic transplantation of splenic tissue into the soft tissue of the body wall rather than the usual peritoneal location, and, second, the implant after having been present for a considerable length of time, suddenly began to enlarge in what may have been a response to a malarial infection.

#### CASE REPORT

In August, 1940, J. M. M., colored male, about 52 years of age, was shot through the lower portion of the left chest. The bullet passed through the left lower thoracic wall in an anterior-posterior direction and fractured the ninth and tenth ribs in the posterior axillary line as it passed through the chest wall. There was considerable hemorrhage from the wound of entrance and exit, but never any hemoptysis or positive x-ray evidence of hemothorax, pneumothorax, or pneumoperitoneum. Note is made, however, that breath sounds were not audible over the left lower chest. Study of x-rays made at this time reveal no visible change in the diaphragm which might be construed as evidence of injury. The patient was discharged from the hospital after six days, without operative treatment.

In December, 1941, the patient again appeared at the hospital with a chief complaint of "vomiting blood and pain in stomach." Three weeks before the date of this admission he was kicked by a mule over the left costal margin, anteriorly. He immediately vomited blood-streaked material. He was not incapacitated, however, and worked until one day prior to admission. On this day he lifted a heavy weight and experienced a tearing sensation in his abdomen followed by constant pain and frequent vomiting of blood-streaked vomitus.

Physical examination revealed absence of breath sounds on the left side below the fourth interspace and a tympanitic percussion note over the left lower chest. X-ray examination (after introduction of stomach tube) revealed a large diaphragmatic hernia on the left with herniation of the stomach up to level of the second interspace. Fracture of the tenth rib in the posterior axillary line with considerable displacement of the fragments was also mentioned. The fracture corresponded to that described in August, 1940, as a result of the bullet wound. The ninth rib was well healed, but there had been almost no change in the tenth rib.

The patient was operated upon, and a large rent in the left posterior diaphragm was repaired. The left chest contained transverse colon, two-thirds of the stomach, and the spleen. It is stated in the operative note that the spleen was adherent to the diaphragm and posterior aspect of the parietal pleura near the vertebral column and had to be removed before the hernia could be repaired. Personal communication from the surgeon and a study of the surgeon's notes indicated that the entire spleen was removed and seemed to be intact, although the upper portion was firmly adherent to the diaphragm and posterior portion of the pleura as mentioned above. The surgeon also stated that the spleen was of average size and shape, but that there were many dense, white, somewhat depressed scars across the organ as if lacerations had occurred in the past. After a stormy recovery, he was discharged in good condition on the twenty-third postoperative day. At the time of this admission a note was made that a small, tender swelling was present in the chest wall at the level of the ninth or tenth rib in the posterior axillary line. This nodule appeared to be in the scar of the bullet wound which was received in August, 1940.

The last admission was on Sept. 15, 1942, at which time the patient complained of soreness and gradual increase in size, during the past three or four weeks, of the small mass in the left posterior axillary line which had been noted on the previous admission. He also complained of vague paresthesias along the lower portion of the left chest extending anteriorly to the epigastric region.

Physical examination revealed a firm, ovoid mass about the size of a hen's egg at the level of the tenth rib in the posterior axillary line. X-ray of the area revealed nonunion of the rib at the site of the old injury and little change in the fragments since the time of the original fracture in August, 1940. Clinical opinion was divided as to whether the condition was that of osteomyelitis or malignant or benign neoplasm.

Laboratory findings on this last admission were: hemoglobin, 13.8 Gm.; red blood cells, 4,450,000; white blood cells, 9,800. The differential count was not remarkable.

Upon the day of admission to the hospital, Sept. 15, 1942, the patient had a sudden rise in temperature to 104° F. and a hard chill at 4 P.M. The temperature rapidly fell after the chill and remained normal or slightly subnormal all the following day. On Sept. 17, 1942, at 4 P.M. the temperature again rose above 105° F., and another hard chill was experienced. On this day malarial parasites were demonstrated in the peripheral blood. Tertian ring forms and gametocytes were described. Quinine sulfate was immediately started, and temperature remained normal until the day of operation, Oct. 8, 1942. Questioning of patient elicited the statement that he had an episode thought to be malaria about ten to twelve years ago, but had no chills or fever in recent years and none immediately before admission to hospital.

On Oct. 8, 1942, the tumor mass was excised with a portion of the tenth rib. The rib appeared to be surrounded by the tumor which was described as "soft tumor mass protruding around the rib and in the soft tissues of the chest wall."

During the operation, the pleural cavity was entered, the diaphragm was accidentally penetrated, and the peritoneal cavity exposed; but the opening was small and no observations

on the peritoneum were made. The opening in the diaphragm was sutured and the wound was closed.

Postoperative course was not remarkable except for moderate elevation of temperature which began the day of operation and persisted for nine days. A single exception to the above was a chill with temperature of 104° the second postoperative day. The patient was discharged in good condition, Oct. 24, 1942.

*Pathologic Examination of the Operative Specimen.*—The specimen consisted of a fragment of broken rib with an attached soft tissue mass. The soft tissue measured 3 x 2 cm. in greatest diameters. It did not appear to arise from the rib itself but lay between the ends of the fragments and around the fracture site. The nodule was well encapsulated. Upon gross examination it was described as red, soft, and very friable.

Microscopic examination of the soft mass revealed well-defined splenic tissue. The pulp was not striking in appearance, except for some fibrosis and rather conspicuous sinusoids. Malpighian bodies were numerous and bulky with large germinal centers.

Trabeculae were present in numbers and appearance suggestive of normal spleen. The capsule on the surface was not thickened and resembled a normal splenic capsule.

Beneath the capsule in pulp there were two small areas of hemorrhage showing early organization and containing a few lymphocytes. Scattered throughout the pulp, for the most part in macrophages, were large quantities of brown pigment. All of the pigment was iron-containing, as determined by the ferrocyanide reaction. No noniron-containing pigment was seen. The gross specimen was discarded before photographs or sections of the bone at the fracture site could be made.

*Discussion.*—The evidence seems to justify the assumption that this is a case of traumatic implantation of splenic tissue in the chest wall at the costophrenic angle.

It seems probable that the bullet in August, 1940, tore through the upper portion of the spleen, lower lateral portion of the left diaphragm at the costophrenic angle, and then passed through the body wall, fracturing the ninth and tenth ribs. In its passage, the bullet may have carried a fragment of splenic tissue through the extreme lower lateral portion of the diaphragm near the attachment to the chest wall and left it lying between the fractured ends of the tenth rib in the body wall where it persisted as an autoplasmic transplant, perhaps explaining in part the nonunion of the fracture.

It is possible, of course, that this portion of displaced spleen maintained some attachment to the main mass of splenic tissue for a time, possibly until the rent in the diaphragm healed. It may be, too, that small fragments yet remain trapped in scarred diaphragm and perhaps in the splenic bed. None were described in the course of the operation for repair of the diaphragmatic hernia, however.

While it is not possible to say from either histologic or clinical evidence that the implant increased suddenly in size as result of malaria, it is hard to escape this impression in view of the order of development of events. It is probably safe to assume that this patient's malarial inoculation took place not less than fourteen days prior to his admission to the hospital. Boyd, Stratman-

Thomas, and Muench<sup>1</sup> have pointed out that the gametocytes of *Plasmodium vivax* have not been observed prior to the fourteenth day following inoculation, and may be later in appearance. The rather rapid and sudden increase in size of the splenic nodule may be significant. Coggeshall<sup>4</sup> has shown that the rate of the spleen increase after the first intravenous injection of *Plasmodium knowlesi* in monkeys followed a straight line until twenty-four hours before death. A much greater and more rapidly developing splenomegaly was noted in animals in which a chronic infection existed. Such animals, when injected with large numbers of homologous parasites, showed a rapid increase in size of the spleen as compared with primary infections, followed, however, by a fairly rapid decrease. It is not at all unlikely that the patient in the case described in this paper had chronic malaria.

In this case it should be noted that the splenic pulp, capsule, trabeculae, and Malpighian bodies appear to be like normal splenic architecture in every respect. The various reports in the literature differ somewhat in the description of the autograft nodules as regards the resemblance to normal spleen. One gains the impression, however, that none depart very far from the usual appearance. In one of the cases described in the literature<sup>11</sup> it was noted that, although the splenic pulp, in implants was easily recognized, it was not considered to be identical in detail, and in only two nodules were there structures which might be an attempt at splenic corpuscle formation. Lee<sup>7</sup> indicated that the implants in his case were like normal spleen with pulp and Malpighian bodies. Buchbinder and Lipkoff<sup>2</sup> noted a case in which the transplants resembled normal spleen except for paucity of lymph follicles and trabeculae and an occasional atypical blood vessel relationship. It may be that the degree of similarity of the implants to the normal splenic tissue depends upon the size and components of the original implant. If all components of the spleen are represented in the implant, one might expect it to duplicate the histology of normal spleen. If, on the other hand, only pulp elements in very small fragments are scattered, it would seem possible that other structures might be lacking or scarce in number, although it is believed that reticular cells of splenic pulp are capable of differentiation into Malpighian bodies as well as pulp elements. In most of the recorded cases there has been extensive tearing of the spleen, with hemorrhage, and, presumably, wide distribution of tiny pulp fragments over the peritoneum. In the case described here, however, a large possibly solitary mass is believed to have been displaced. That the presence of all components of adult spleen in implants is not necessary to produce grafts similar to normal spleen is suggested by work of Calder<sup>3</sup> on experimental animals. He observed that in experimental implants all except a narrow rim of tissue at the periphery of the implant becomes necrotic and that the necrotic center is then replaced by proliferation of undifferentiated reticular cells which survive at the edge. He feels that most elements of spleen can be derived from these cells and that the essential factor in growth of splenic grafts is survival and proliferation of reticulum cells.

The possibilities of experimental subcutaneous autografts of spleen have been explored and appear to offer no serious problems. According to Marine and Manley<sup>8</sup> autografts of spleen introduced beneath the subcutaneous fascia

of the anterior abdominal wall in rabbits seldom fail to survive. They state, however, that although an autograft may survive or even grow slightly, it does not show really extensive growth unless the spleen is partially or completely removed. Their transplants which grow or survive are said to have all the general characteristics of normal spleen, both as to number of component structures and their relationship to each other. It appears that no experiments were carried out by them relative to reaction of implants to infection, but they noted that in several cases where animals died of pneumonia, the grafts were congested, and in one case, the transplants were soft, showed increased pulp cells; and they suggest that transplants might react to infections as the intact spleen does. Perla and Gottesman<sup>10</sup> indicate that in many cases, splenic transplants in rats react to protect the animals against *Bartonella muris* anemia. It is interesting that typical tubercles were described in several implants by Shaw and Shafi as an indication that this common finding in intact spleens is also noted in implants. They state, however, that no bilharzial pigment was present in implants, although the patient showed infestation. (It will be recalled that only iron-containing pigment was observed in the case reported in this paper).

It has been suggested by Morrison, Lederer, and Fradkin<sup>9</sup> that accessory spleens remaining after splenectomy may account for some of the failures of operation in certain conditions, such as thrombocytopenic purpura. With this in mind, it would seem probable that implants of spleen might also maintain functional capacity similar to the intact normal spleen, especially when that organ has been removed. It is suggested by the case described here that malaria may be capable of causing enlargement in autografts of the spleen.

*Summary and Conclusions.*—A case is reported of bullet wound through lower left chest at about the level of the tenth rib in the posterior axillary line. About fifteen months later the patient suffered a rupture of the diaphragm with diaphragmatic hernia; this was repaired about three weeks after the accident. At time of repair the apparently intact spleen was found adherent to the lower surface of the diaphragm and to the posterior chest wall near the spine. It was dissected free and removed to facilitate closure of the rent in the diaphragm. About nine months after operation, a small subcutaneous mass in the left posterior axillary line, which was known to be present in the scar of the bullet wound prior to the operation for repair of the diaphragmatic hernia, increased rather rapidly in size and became painful. Shortly after change in size was noted, the patient developed typical malaria. Operation revealed a mass of characteristic splenic tissue lying in chest wall between the broken ends of the tenth rib. While it is not felt that a positive diagnosis of "malarial" splenomegaly in the autograft of spleen can be made, this possibility is indicated.

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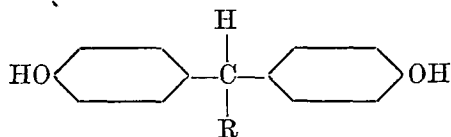
# THE BACTERICIDAL ACTIVITY OF SOME DI-(HYDROXYPHENYL) ALKANES\*

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## INTRODUCTION

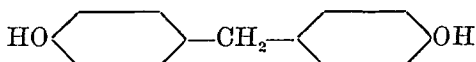
THE bactericidal properties of phenol and phenolic compounds have been the subject of considerable intensive research. On the other hand, bactericidal investigations of series of di-(hydroxyphenyl) alkanes have been somewhat limited.

Harden and Reid<sup>1</sup> studied, among other compounds, a series of the type:



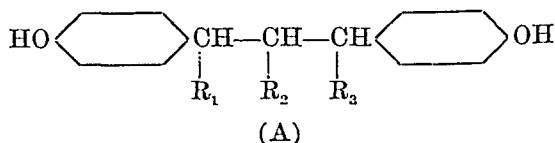
where R may be hydrogen or an alkyl group (from methyl to n-hexyl). Their results showed increasing germicidal activity, against *Staphylococcus aureus*, with increasing length of the aliphatic chain.

Richardson and Reid<sup>2</sup> continued these studies on several  $\alpha, \omega$  derivatives of the type:



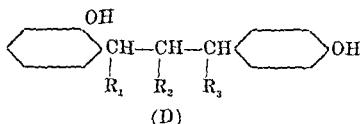
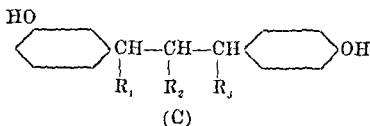
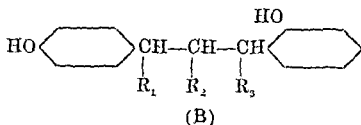
in which the aliphatic chain varied in length from  $\text{CH}_2$ , as shown, to  $\text{C}_{10}\text{H}_{20}$ . These compounds were much less soluble than the corresponding  $\alpha, \alpha$  derivatives, and they were able to obtain maximum killing dilutions only for the first four members of the series. Lengthening the aliphatic chain was found to increase bactericidal power against *S. aureus*.

As the result of some organic syntheses, in this laboratory, 23 di-(hydroxyphenyl) alkanes were made available for bactericidal testing. The basic formulas for the compounds discussed in this report, based on the position of the hydroxyl groups, are as follows:



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$\text{R}_1$ ,  $\text{R}_2$ ,  $\text{R}_3$  may be hydrogen or an organic radical.

For convenience, these compounds are divided into series based on the structure of the aliphatic chain. Series I includes compounds of basic formulas (A), (B), (C), and (D). All the compounds in the other series have the basic formula (A). Chemical investigations of monosubstituted derivatives of 1, 3 di-(p-hydroxyphenyl) propane have been presented by Stuart and Tallman.<sup>3</sup> Chemical studies on the more highly substituted derivatives are to be reported.

#### METHODS

Stock solutions of the compounds were prepared, in suitable concentrations, in 95 per cent ethyl alcohol, by volume. Dilutions were made from the stock with sterile distilled water. Maximum bactericidal dilutions were obtained in the presence of 20 per cent alcohol, by volume, in all instances, since it is known that the activity of phenolic compounds may be influenced by the concentration of alcohol present.<sup>4</sup> A 20 per cent concentration of ethyl alcohol, by volume, did not kill the test organisms within the time limit used.

The test organisms were *S. aureus* 209 and *Eberthella typhosa* grown in F.D.A. nutrient broth. To 5 c.c. of diluted compound, at 20° C. there was added 0.5 c.c. of 18- to 24-hour growth of culture. After standing at this temperature for ten minutes, transfer was made with a 4 mm. loop (No. 23 B. & S. gauge wire) to 10 c.c. of sterile nutrient broth. Subcultures were incubated at 37° C. for forty-eight hours and maximum killing dilutions determined. Tests made for bacteriostatic properties revealed that it was not necessary to further transfer the subculture.

#### RESULTS

The bactericidal dilutions, against *S. aureus* and *E. typhosa*, for the compounds tested are shown in Table I. It is noted that bactericidal activity against *S. aureus* increases, in the four series of compounds, as the molecular weight of

It is interesting to note that the series of compounds tested have other physiologic properties in so far as they are able to bring about estrogenic responses.<sup>6</sup>

#### SUMMARY

Twenty-three di-(hydroxyphenyl) alkanes have been tested for bactericidal activity. In general, they exhibit increased killing action, in vitro against *S. aureus*, with increasing length of the alkyl chain. The susceptibility of *E. typhosa* is at a maximum with the n-butyl derivative. Generalizations which have been applied to other series of phenols, regarding the relation of bactericidal activity to chemical structure, are found to be valid for the series of compounds tested.

Grateful acknowledgement is made of the advice and encouragement given by Dr. R. C. Tallman, Director of Research, in this problem and also for supplying the compounds tested.

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# A STUDY OF OILS USED FOR INTRAMUSCULAR INJECTIONS\*

## A STUDY OF THE PHYSICAL, CHEMICAL, AND BIOLOGIC FACTORS

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ALL physicians active in the clinical practice of medicine find it convenient to administer drugs in oil by intramuscular injection. With the increasing use of water-insoluble drugs, this route of administration is becoming more frequent. While it is true that most of the oils placed on the market at the present time are fairly satisfactory, all of us sooner or later find patients who react unhappily to some of them. Because of such an experience in our own clinic, we undertook to investigate the factors which should guide a physician in his selection of the oil vehicle for such medication.

The ideal oil should meet the following requirements:

*Chemically* it should be a stable oil, neutral in reaction, i.e., containing no excess acid. It should not react with the medication to form toxic degradation products.

*Biologically* it should be inert and nonirritating. This oil should be essentially free of antigenic properties and be rapidly absorbed from living tissue leaving no residue.

*Physically* it should be a good solvent or dispersing medium. It should not be too viscid to pass readily through the needle. The extremes of temperature encountered in sterilization (200° F.) and freezing (-10° to -20° F. of doctor's car in winter) should not materially alter any of the above features.

In searching through the literature, very little information is available regarding these data on the oils in common clinical use. Most of the articles concern themselves with specific examples of allergic reaction or hypersensitivity to some particular oil. While these cases are not too uncommon, they are a specialized group of reactions and were not considered in this study.

Emery et al.<sup>1</sup> working with rats studied the absorption of stilbestrol and theelin from intramuscularly injected sesame, peanut, castor, and olive oils. They found oil retention cysts in these rats ranging in size from microscopic to 1 or 2 mm. in size. Emery et al. found that oils differed somewhat in the cyst formation which they incited, and these authors recorded the following order of increasing tissue reaction: peanut, olive, sesame, and castor.

Deanesly and Parkes<sup>2</sup> studied the absorption of oil following subcutaneous injection. They report that in rats and mice, olive oil is absorbed most rapidly, that sesame and castor oil are absorbed with increasing difficulty, and that castor

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oil remains in tissue almost indefinitely. Chart 1, modified from these authors, graphically presents their data.

These two reports suggest that castor oil produces the most marked reaction and persists longer than other oils and that olive and sesame oils are the better oils for clinical use.

Dr. E. A. Sharp<sup>3</sup> has made available to us, through personal communication, the results of observations on the inflammatory reaction produced by the intramuscular injection of olive, sesame, and peanut oils. Chart 2 is prepared from such data and shows that olive and sesame oils produced the least inflammatory changes in muscle tissue and were the most readily absorbed. Peanut oil produced the most marked inflammatory change.

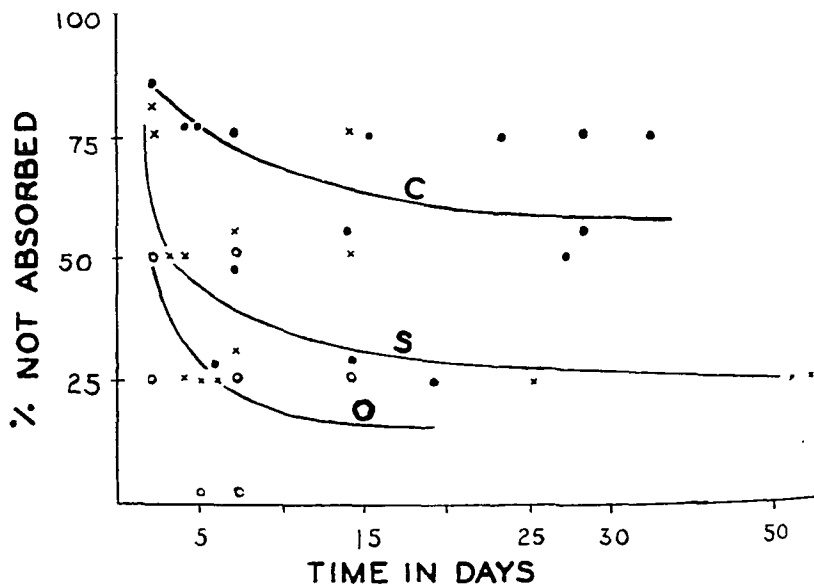


Chart 1.—The absorption of oil from subcutaneous injection. Arranged from data published by Deanesly and Parkes.<sup>2</sup>

Olive oil O

Sesame oil X

Castor oil •

#### METHOD

Because of the scarcity of material in the medical literature on this subject, we set up a project designed to assist us in the selection of the best oil for intramuscular injection. Four commonly used oils (corn, cotton seed, sesame seed, and peanut) were selected for study. Samples of these oils and their chemical and physical properties were made available to us through the courtesy of Dr. F. S. Bukey.

The antigenic properties were investigated in the following manner. Twenty patients received at weekly intervals two injections of each of the four oils to be considered. Approximately two months later these patients were tested by the patch and intracutaneous test techniques according to standard specifications.<sup>4</sup>

The biologic reaction was investigated by injecting oils intramuscularly into the large extremity muscle of experimental animals (rats and rabbits). Every

possible care was taken during the injection of the oil to obtain satisfactory antisepsis and to deliver as nearly as possible pure sterile oil. Injections were made at right angles to the surface, as near the center of the muscle as possible, and the area was gently massaged. These animals were sacrificed at varying time intervals of from one to nineteen days. The muscle was dissected free and fixed in 10 per cent formalin. The tissue was then cut, and frozen sections were prepared for fat stain with scharlach R. The balance of the muscle was then run through dehydrating alcohols, xylol, into paraffin sections and stained with hematoxylin and eosin.

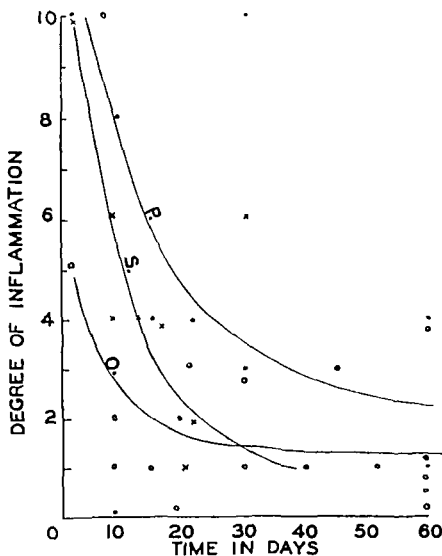


Chart 2.—Inflammation induced in muscle by injected oil. Arranged from data furnished by Dr. Sharp.  
Olive oil O      Sesame oil X      Peanut oil •

#### RESULTS

In Tables I, II, and III will be found a summary of the data observed. The chemical and physical properties of these oils are shown in Table I. Corn oil showed slightly less viscosity than the other oils at room temperature but was definitely less viscid at 3° C. Corn oil remains fluid down to temperatures of -10° to -15° C. (solidification point). This becomes significant for physicians who carry their bags in their cars in the winter time. All of the oils tolerated sterilization temperatures satisfactorily, smoking temperatures being above sterilization temperatures in all cases. The acids found in these oils are the result of hydrolysis. There is considerable variation of the acid value in different

samples of each oil. Apparently the source and handling of the material are more important than the type of oil when this factor is considered. Several pharmaceutical laboratories have added chemical agents as preservatives, either as bacteriostatics or antioxidants. Some of these agents have proved quite irritating clinically. Hydroquinone was found as an antioxidant in one manufacturer's product. This was effective in preventing the deterioration of the active ingredient, but proved very irritating clinically in some cases, especially if a small amount of oil was deposited subcutaneously.

TABLE I  
THE PHYSICAL AND CHEMICAL PROPERTIES OF THE OILS TESTED

		COTTON SEED	SESAME SEED	CORN	PEANUT
"SMOKING TEMPERATURE" (C°)		235°	212°	243°	220°
Solidification Point (C°)		-12° to -13°	-4° to -16°	-10° to -20°	-3°
Specific Viscosity (Oil/Water)	23° C.	33.9	31.8	29.7	35.5
	3° C.	82.7	71.6	63.9	Semisolid
Acid Value		2.5 to 7.3*	3.0 to 1.4	2.5	0.12 to 0.3

\*One sample had an acid value of 21.6.

TABLE II

PATIENTS GIVEN TWO SENSITIZING INJECTIONS OF A POOLED SAMPLE OF ALL FOUR OILS AT ONE WEEK INTERVAL. SIX TO EIGHT WEEKS LATER THEY WERE TESTED FOR SENSITIVITY BY PATCH AND INTRACUTANEOUS INJECTION OF EACH OIL, AND THE REACTION READ AT 24°, 48°, AND 72°.

PATIENT	COTTON SEED	CORN	SESAME SEED	PEANUT
	24° 48° 72°	24° 48° 72°	24° 48° 72°	24° 48° 72°
L	- + +	- - -	- - -	+ + +
C	- - -	- - -	- - -	- - -
U	- - -	- - -	- - -	- - -
S	- - -	- - -	- - -	- - -
G	- - -	- - -	- - -	- - -
K*	- + +	- - -	- - -	- + +
T	- - -	- - -	- - -	- - -
D	- - -	- - -	- - -	- - -
H	- - -	- - -	- - -	- - -
C	- - -	- - -	- - -	- - -
B	- - -	- - -	- - -	- - -

\*On fifth day (120 hrs.) there was erythema about all sites of injection.

Of the twenty patients studied for the antigenic effect of these oils, three complained of pain on the second sensitizing injection; the others offered no complaint. Patch and intracutaneous tests were completed on eleven patients, and the results are found in Table II. Reactions in general were uncommon. Definite conclusions cannot be drawn from this small series, but it would appear that sesame and corn oil are less antigenic than the cotton seed and peanut oils.\*

On studying the tissues microscopically, we observed a typical trend of reactions. Within twenty-four hours there was a moderate increase in round cells and the oil seemed to be diffusely scattered through the muscles. It had

\*No attempt is made to study the mechanism of this sensitization. It has been suggested that it results from small amounts of protein in the oil.



Fig. 1.



Fig. 2.

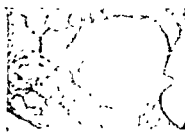
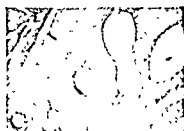
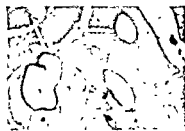


Fig. 3.



A.



B.



C.

Fig. 4.

Fig. 1.—Peanut oil reaction seen at 24<sup>h</sup>—low power.

Fig. 2.—Coin oil reaction seen at five days—low power.

Fig. 3.—Fat stain—peanut oil 4 days—low power.

Fig. 4A, Sesame oil at ten days, low power.

B, Peanut oil at ten days, low power.

C, *ibid.*, high power.



TABLE III

THE MICROSCOPIC STUDY OF TISSUE REACTION TO INTRAMUSCULARLY INJECTED OIL

RABBIT				
DAY	COTTON SEED	SESAME SEED	CORN	PEANUT
1	Many leucocytes. Oil along fascial planes. (No cysts in spec.)	Oil in cysts and along fascial planes. Very few leucocytes.	Oil in cysts. Very few leucocytes about cysts.	Oil in cysts. Many leucocytes.
4	Oil along fascial plane and in cysts. Leucocytes about some of the cysts.	--	Oil in cysts. No leucocytes.	Some of cyst walls very thick. Mod. leucocytes.
11	Oil cysts. Moderately thick walls. No leucocytes.	Thin-walled cysts.	Very thin-walled oil cysts. No leucocytes.	Some of cyst walls very thick. Many leucocytes.
14	Thick-walled cysts. No leucocytes.	--	Very thin-walled oil cysts. No leucocytes.	Some of cyst walls very thick. No leucocytes.
RAT				
1	Oil beginning to be encysted. Few leucocytes.	Very few leucocytes. Oil dispersed.	Very few leucocytes. Oil dispersed.	Many leucocytes, edema. Oil dispersed.
4	Mod. encystment. Wall mod. thick. Some leucocytes.	Oil encysted. Thin walls. Few leucocytes. No edema.	Clear cysts with very thin walls. Few leucocytes.	Part of oil encysted. Heavy leucocytes and edema. Some muscle necrosis. Wall surrounded by leucocytes.
10	Encysted oil. Mod. thick wall. Few leucocytes.	Cyst wall mod. thick. Mod. leucocytic reaction.	Thin-walled cysts. Leucocytes nearly gone. Slight reaction.	Oil cysts persist with heavy leucocytes and edema.
14	Oil cysts persist and mod. thick wall. Few leucocytes.	Small thin-walled Mild leucocytes.	Cysts smaller. Leucocytes nearly gone. fibroblasts in cyst wall.	Similar to above.
Severity of reaction	3	2	1	4

a tendency to accumulate along fascial sheaths, but was also distributed between muscle bundles. (Fig. 1). In two or three days the oil tended to accumulate into small droplets, and around these droplets appeared a layer of fibrin with a large epithelioid type of cell. The accumulation of leucocytes and wandering cells was peripheral to this oil cyst (Fig. 2). Fig. 3 is of tissue prepared for the demonstration of fat and is stained with scharlach R; this demonstrates that these spaces are actually oil cysts. The leucocytic reaction reached a peak between days 3 and 6 and then tended to subside slowly. The rate and intensity of these changes varied in the different oils. In Table III are recorded our observations. Based on these factors we have attempted to grade the tissue reaction to these oils. In general, all of the oils formed many microscopic cysts throughout the involved muscle. As noted in this chart, there is a qualitative and quantitative difference in response. Fig. 4 compares the microscopic picture of corn oil and peanut oil as seen at ten days.

In general, corn and sesame oil produced the least amount of reaction, as judged by the amount of fibrin deposited, and leucocytic infiltration for the

shortest period of time. The persistence of oil cysts could not be determined from this short period of observation; they did, however, seem to be smaller in corn and sesame oil animals. Peanut oil produced the most marked reaction; and cotton seed oil was in between these, showing somewhat greater variation from animal to animal.

#### CONCLUSION

1. The criteria for selecting an ideal oil for intramuscular injection have been suggested. These include physical, chemical, and biologic specifications. Using these criteria, corn, peanut, sesame seed, and cotton seed oils were studied.

2. The physical and chemical properties of these oils as they apply to their use in medicine have been reviewed and evaluated.

3. The antigenic properties of these oils were tested; sesame seed and corn oil did not produce sensitivity by this technique, while cotton seed and peanut oil produced reactions.

4. The reaction of muscle to these oils was studied, and the following factors have been used in evaluating the intensity of this reaction: (a) accumulation of leucocytes, (b) formation of oil cysts, and (c) disposition of fibrin.

5. By these criteria *sesame* and *corn* oil are superior to peanut and cotton seed oil for intramuscular injection, for they are: (a) more suitable physically and chemically for this purpose, (b) more quickly absorbed from tissue, (c) less antigenic, and (d) less irritating to tissue.

We wish to express our appreciation to Dr. J. P. Tollman, Professor of Clinical Pathology, who assisted in evaluating the tissue reaction.

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# BARBITURATE INTOXICATION AND PICROTOXIN TREATMENT

## PRESENTATION OF A CASE

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DESPITE lack of complete information, reports on barbiturate poisoning indicate that it is a problem of important magnitude. Hambourger,<sup>1</sup> at the request of the Council on Pharmacy and Chemistry of the American Medical Association, has made a study of the promiscuous use of the barbiturates. He concluded:

1. More than 1,200,000,000 grains of barbituric acid derivatives were sold in the U.S.A. in 1936.

2. The number of suicidal deaths by the barbiturates in the U.S.A. in 1936 was 300.

3. The number of suicides by barbiturates has shown a definite upward trend during the past decade.

Statistical reports of the Medical Examiner's Office of the City of New York revealed the number of suicides by barbiturates, accidental and undetermined, in the City of New York alone, to be 32 cases for the year 1937; 42 cases for the year 1939; 48 cases for the year 1940; and 60 cases for the year 1941. Accurate statistics as to the number of persons poisoned who did not die are not available, as physicians do not report those cases.

Perusal of foreign literature reveals that the use of barbiturates is as common abroad as in the U.S.A.<sup>2</sup> Thus the widespread use of barbiturates by the layman has inevitably led to many instances of accidental overdosage or suicidal poisoning. It is therefore imperative that the general practitioner, as he is the one who is first called, be familiar with the symptoms of acute intoxications, as well as cognizant of a suitable and energetic antidote.

The symptoms of barbiturate poisoning vary with the amount of drug taken. When absorption is gradual, confusion, ataxia, difficulty in swallowing, vomiting, and motor excitement occur. Diagnosis is apt to be difficult. Any patient found in deep coma, with shallow rapid respiration, fixed miotic pupils, corneal reflexes abolished, absent reflexes, thready pulse, and low blood pressure, should suggest the possibility of barbiturate poisoning. Energetic therapy is essential; the aim of the treatment is to combat the action of the drug by antagonistic drugs and supportive therapy. Table I contains approximate fatal doses for the commoner barbiturates.<sup>3</sup>

Picrotoxin is the active principle of several plants, notably *Anamirta paniculata* or "fish berries."<sup>3</sup> Its use as an antidote in barbiturate intoxications was first suggested by Maloney, Fitch, and Tatum<sup>4</sup> on the basis of animal experiments. Picrotoxin gained the attention of the clinicians as a result

of the pioneer work and studies of Maloney and his associates,<sup>5</sup> and Linegar and his co-workers.<sup>6</sup> Murphy et al.<sup>7</sup> reported the first clinical attempt to administer picrotoxin in adequate doses in acute barbiturate poisoning. A number of clinical cases<sup>8-12</sup> have been reported which seem to substantiate the animal evidence that picrotoxin is an effective physiologic antidote for barbituric acid intoxications in human beings.

Picrotoxin stimulates the medullary centers and cortical centers. The respiratory and vasomotor centers and the autonomic centers are also stimulated. There is a hyperactivity of the skeletal muscles and some myoclonic movements. In toxic doses, it causes nausea, vomiting, cold sweat, diarrhea, slow pulse, clonic convulsions, and paralysis. Picrotoxin, injected intravenously, disappears rapidly from the blood and could not be demonstrated there or in the liver or muscles after two hours.<sup>13</sup>

It has a narrow margin of safety. However, clinical evidence indicates that in barbiturate poisoning much higher doses may be used. It should be administered by divided doses. The amount and interval should depend upon the response of the patient. The immediate object is to maintain adequate respiratory and circulatory function by the stimulant. Bleckwenn et al.<sup>14</sup> in a series of clinical observations found approximately one mg. of picrotoxin was an antidote for approximately 30 to 40 mg. of pentobarbital sodium.

TABLE I

COMPOUND	SEVERE INTOXICATION, BUT GENERALLY WITH RECOVERY	GENERALLY FATAL
Allonal	10 Gm.	More than 15 Gm.
Amytal	1.5 to 2 Gm.	2 to 3 Gm.
Barbital	3 to 10 Gm.	5 to more than 20 Gm.
Dial	2 to 2.5 Gm.	More than 2.5 Gm.
Nembutal (Pentobarbital Sodium)	More than 1 Gm.	More than 2 Gm.
Pernocton	0.5 to 1 Gm.	More than 1 Gm.
Phanodorm	1.2 Gm.	More than 10 Gm.
Phenobarbital	4 to 7 Gm.	6 to 9 Gm.

It is the purpose of this communication to report a case of pentobarbital sodium (nembutal) intoxication treated by picrotoxin with recovery. The patient swallowed 3 grams of the drug which is considered a lethal dose.

#### CASE REPORT

The patient, a 24-year-old nurse, was first seen in deep coma at 8 P.M. on May 13, 1943. Her color was dusky, pupils miotic and nonreacting. All her reflexes, including the corneal, were absent. Her heart rate was rapid and sounds were of feeble quality; the pulse small, rapid, and thready, rate 124 per minute; the blood pressure 90/50. The respiratory rate was 30 per minute, labored but not stertorous; temperature 96.4° F.

The family revealed she had threatened suicide. Later, on recovery, patient verified that she had swallowed three grams of nembutal at about 4 or 5 P.M. that afternoon.

May 13, 1943, *Treatment Administered:*

(a) *The immediate treatment:*

1. Intravenous injection of 1.5 c.c. size coramine ampules every fifteen minutes for six doses, followed by
2. Six intramuscular injections of metrazol ampules (1 c.c.) at half-hour intervals.
3. Gastric lavage

4. Body heat applied

5. Change of patient's position with removal of tracheal secretion.

The results with the therapy were negative, and the patient's condition remained critical. It was felt that we were dealing with a severe case of deep barbiturate poisoning, and therefore treatment with picrotoxin was instituted at 1 A.M., i.e., about five hours after first seeing the patient.

*(b) Picrotoxin Therapy:*

May 14, 1943, 1 A.M.: 2 c.c. of a 0.3 per cent (6 mg.) solution of picrotoxin was given intravenously.

1:15 A.M.: 2 c.c. of 0.3 per cent (6 mg.) solution of picrotoxin was given intravenously.

1:30 A.M.: 1 c.c. of 0.3 per cent (3 mg.) solution of picrotoxin was given intravenously.

1:45 A.M.: 1 c.c. of 0.3 per cent (3 mg.) solution of picrotoxin was given intravenously.

2 A.M.: 1 c.c. of 0.3 per cent (3 mg.) solution of picrotoxin was given intravenously.

The patient at this time began to manifest restlessness, winced when her skin was pinched hard, and dribbled her urine. Temperature rose to 98.2; pulse rate diminished to 110; blood pressure 100/60; respiratory rate was 24.

Picrotoxin was continued at half-hour intervals. During this time other adjuvant measures, such as (1) continued body heat, (2) maintenance of open airway; and (3) changing of patient's position, were constantly carried out.

May 14, 1943, 6 A.M.: Corneal reflexes returned; there were twitchings of the tongue and lips. Heart sounds improved, the pulse rate was 100 and of good quality. The lungs were clear; lips were dry but less cyanotic. Injections with picrotoxin were continued at 1 c.c. doses intramuscularly at 45-minute intervals.

7 A.M.: Temperature began to rise and reached 100.6; pulse rate increased to 120, but was of good quality; and the respiration was 29. Lungs were clear. It was felt that the patient's temperature was due to dehydration.

8 A.M.: Temperature was 101.2; pulse rate, 138; and the respiratory rate, 28.

Patient showed increasing restlessness; blood pressure rose to 120/70; heart sounds were of good quality; and picrotoxin was continued intramuscularly at 45-minute intervals in 1 c.c. doses. Pupils reacted to light and were of normal shape.

9 A.M.: 2000 c.c. of 5 per cent glucose in saline intravenous clysis, high colonic irrigation, followed by retention enema of warm coffee.

11 A.M. to 2 P.M.: Reflexes returned and coma seemed less severe.

1. Continued 1 c.c. of picrotoxin intravenously at 45-minute intervals.

2. Continued intravenous drip at the rate of 30 drops per minute.

2 P.M.: Patient vomited green mucus, followed by loose bowel movement; temperature fell to 100.6, pulse rate was 100, and the respiration rate was 20. Patient grunted and moved when pricked.

At this time it was decided to stop picrotoxin injections with the view of ascertaining whether recovery would continue without analeptic medication. Caffeine-sodio-benzoate ampules, 0.5 grams intramuscularly, was substituted at half-hour intervals.

4:30 P.M.: A definite depression was noted, patient became quite immobile, respiration labored, and the pulse rate rose to 119; the temperature became normal, 98.6.

One c.c. intravenous injection of picrotoxin was resumed at 15-minute intervals for two doses. Remission was immediate, twitchings were noted; there was spontaneous bowel evacuation with vomiting of bile.

5 P.M., May 14, 1943, to 4 A.M., May 15, 1943: Picrotoxin continued intramuscularly at 45-minute intervals.

May 15, 1943, 4 A.M.: Patient showed convulsive movement of head, and legs began rolling from side to side. Picrotoxin was stopped.

4 A.M. to 5:45 A.M.: No medication. Patient quiet, physical condition good.

5:45 A.M.: Picrotoxin given intramuscularly at 1 hour intervals for two doses.

Patient became active, kicking covers off body, lifting head, waving arms and grunting.

8 A.M.: Patient began to cry, continued to kick, and roll in bed. Stopped picrotoxin therapy. Color good, pulse and heart rate of good quality, rate 90 per minute.

9 A.M.: Patient uttered indistinct sounds; enunciation slow; speech thickened, unintelligible; crying; still unconscious. No medication.

12 M.: Intervals of lucidity and crying spells; quiet sleep; restless muttering; sounds more distinct; speech less slurred.

4 P.M.: Patient perfectly lucid, fully recovered except for weakness.

#### COMMENT

In the case presented certain facts emerge and are worthy of comment. The physical signs indicated severe poisoning from barbiturates. The amount of drug taken was within the range considered fatal. Although high doses of metrazol and coramine were employed in the beginning of therapy, the results were negative. The intravenous use of picrotoxin (21 mg.) in one hour elicited a marked awakening phenomenon. After thirteen hours of continuous picrotoxin therapy, with definite improvement in the patient's condition, it was decided to stop analeptic therapy. The patient was examined two and a half hours later, and definite depression was noted, the pulse rate increased, respiration became labored, etc. Renewal of intravenous injections of picrotoxin brought about immediate remission.

The Council on Pharmacy and Chemistry<sup>15</sup> stated the following:

"The cautious use of picrotoxin in barbiturate poisoning would therefore seem justifiable in cases which can be carefully studied with the view that they may supply sufficient accurate data for which the proper place of picrotoxin as an antidote may be established."

No pretense is made to recommend picrotoxin as the sole antidote in barbiturate poisonings. Mild cases of barbiturate poisoning need not be treated with picrotoxin. There are accumulated sufficient experimental and pharmacologic evidences to establish picrotoxin as an active antidote for patients profoundly depressed by barbiturate poisoning. The present case and a number of clinical cases which have been reported seem to substantiate the experimental animal evidence.

#### SUMMARY AND CONCLUSIONS

1. Statistics reveal a widespread increase in the use of barbituric acid derivatives and a resultant rise in the number of poisonings, intentional or accidental.

2. The diagnosis of barbiturate poisoning is difficult; the symptoms are manifold. The presence of coma with shallow rapid respiration, contracted pupils, absent or diminished reflexes, and feeble and rapid pulse should raise the possibility of barbiturate poisoning.

3. A case of pentobarbital sodium poisoning is reported. The patient was treated by injections of picrotoxin. She received 135 mg. of the drug. The patient recovered after swallowing 3 grams of the drug, considered within the range of a lethal dose.

4. Metrazol and coramine were employed in the beginning of therapy, but there were no clinical signs of improvement until picrotoxin was administered.

5. It may not be amiss to mention that in this instance picrotoxin could not be purchased, although practically every pharmacy in the neighborhood was



called. The drug had to be borrowed from a hospital. It is therefore suggested, in view of increasing barbiturate use in our country, that picrotoxin be stored by druggists.

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## COLD HEMAGGLUTINATION REACTIONS IN TUBERCULOSIS\*

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THE diagnosis of primary atypical pneumonia of unknown etiology is frequently difficult and uncertain. The signs, symptoms, clinical course, and x-rays may vary from case to case, and from season to season. Recently Peterson, Ham, and Finland<sup>1</sup> suggested the possibility that the presence of cold autohemagglutinins might serve as a diagnostic aid in some forms of the disease. However, more extensive research is indicated to establish whether a titer exists above which the sera of patients with atypical virus pneumonia would invariably agglutinate their own cells, and below which no other serum would demonstrate the same phenomenon.

Horstmann and Tatlock<sup>2</sup> investigated the problem still further and employed isohemagglutination methods, using washed type "O" cells. They reported a high incidence of cold isoagglutinins of significant titer in the serum of patients with atypical pneumonia. Their control patients were invariably negative for cold agglutinins.

The present investigation is an attempt to discover the degree of specificity of cold autoagglutinins and isoagglutinins in random control hospital and clinical patients, as well as to study these reactions in patients with early and late tuberculosis. The latter group was chosen because it not only represented another form of pneumonitis, but a form which, in its early stages, conceivably might be confused with atypical pneumonia.

Eighty-two proved cases of tuberculosis were studied. Thirty-three were at the time untreated patients admitted to the tuberculosis wards. Forty-nine were ambulatory, receiving pneumothorax treatment in the outpatient clinic. The ward cases consisted of fourteen with symptoms of six months' duration or less, considered early cases, and nineteen with symptoms of more than six months' duration, considered chronic cases. Six of the former group had symptoms of less than one month duration or no symptoms at all. A control group of eighteen patients from an acute medical ward and twenty-four patients chosen at random from the outpatient clinic were included.

### METHOD FOR COLD ISOAGGLUTINATION TEST

1. *Preparation of Washed Type "O" Cells.*—A sample of six c.c. of fresh cells from a Blood Bank Flask from which the plasma had been siphoned or ten c.c. of blood from a type "O" donor mixed with 1 c.c. of 2.5 per cent sodium citrate was used. It was centrifuged at 1,500 revolutions per minute for five minutes. The supernatant fluid and layer of white cells were removed by siphon. Twenty-five c.c. of sterile normal saline (0.85 per cent) were added and the mixture was centrifuged again for five minutes. This was repeated three times. If hemolysis occurred, the sample was discarded. The cells were

\*From the Biochemical Laboratories, Kings County Hospital, W. W. Hala, Director of Laboratories.

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packed down well, and a 2 per cent suspension was made by diluting 2 c.c. of the washed cells to 100 c.c. of sterile normal saline solution. The unwashed cells keep for about three days in the refrigerator. The cell suspension must be kept in a refrigerator and may be used until hemolysis occurs.

2. *Procedure for Cold Isoagglutination Test.*—One c.c. of the patient's clear unhemolyzed serum was placed in a test tube containing 3 c.c. of normal saline and mixed. One c.c. of the diluted serum (1:4) was transferred to a small test tube, and 0.1 c.c. of the freshly prepared 2 per cent suspension of human cells was added. The tube was then placed in an ice bath kept in a refrigerator at 1 to 4° C. Readings were taken in six to twelve hours, and the tubes were kept in the ice bath until ready to read. The above procedure was repeated with 1 c.c. of 1:160 and with higher dilutions of the serum. The results were reported as follows:

A. Negative: If on inverting the tube three times, a homogeneous suspension of cells is present.

B. Doubtful: If on inverting the tube three times, very fine particles are present, but disappear within thirty seconds to give a suspension similar to A.

C. Positive: If on inverting as above, there are present many fine persistent particles (1+) to a blood disc which is loosened from the bottom of the tube and which breaks into large coarse particles (4+).

In order to confirm that the positive reactions were typical cold agglutinins, the tubes were allowed to remain at room temperature for an hour or until the agglutinations disappeared. The tubes were replaced in an ice bath in the refrigerator for from two to six hours. The agglutinations always reappeared.

#### PROCEDURE FOR COLD AUTOAGGLUTINATION TEST

One c.c. of clear unhemolyzed serum to be tested was placed in a small tube. The tube containing the clot was inverted several times and 0.05 c.c. of the suspension of the patient's cells was added to the serum tube. The tubes were placed in an ice bath as described under isoagglutination, and the results were read in the same way.

#### COMMENT

In no instance was a positive isoagglutination obtained in a titer of 1:160 or over. One patient in the early tuberculosis group showed a positive test, but no patients with symptoms of one month or less demonstrated a positive reaction. In the control group of ward patients the diagnoses of those reacting positively were hypertensive cardiovascular disease, undiagnosed gastrointestinal disease, and nonspecific colitis. No history of virus pneumonia or tuberculosis was obtained from any of these patients. No positive tests were found in the outpatient clinic control group.

#### RESULTS

The results are reported in Table I.

The present series is not large enough to draw final conclusions concerning the relative correlation of cold autoagglutinins and isoagglutinins with early

and late cases of tuberculosis. It is interesting that no high titers were found in this series, but it is impossible to state with certainty as yet that high titers do not exist in some stage of the tuberculosis disease process. If further investigation of large numbers of patients reveals that those with pneumonitis other than atypical pneumonia invariably fail to develop cold agglutinins of high titer, the test will prove to be of great value in the differential diagnosis of virus pneumonia.

TABLE I\*

PATIENTS	SEX	NUMBER	POSITIVE ISOAGGLUTININS (1:4 DILUTION)		POSITIVE AUTOAGGLUTININS	
			NUMBER	PER CENT	NUMBER	PER CENT
T.B. Wards	M	23	6	26	12	52
	F	10	3	30	6	60
	M+F	33	9	27	18	55
T.B. Clinic	M	26	3	12	9	38
	F	23	5	22	14	61
	M+F	49	8	16	23	47
Control						
Clinic	M+F	24	0	0	2	8
Ward	F	18	3	17	5	28
Total	M+F	42	3	7	7	17

\*The tuberculosis cases were studied through the courtesy of Dr. C. E. Hamilton, Director of the Tuberculosis Services at Kings County Hospital.

It was also noted that not only did autoagglutinins occur more frequently in all groups than did isoagglutinins, but that there was a definitely larger number of positive autoagglutinin tests obtained in the tuberculosis groups than in the control group. More extensive work is indicated to discover whether there is any clinical significance to this observation, or whether this is merely an example of the rather nonspecific nature of cold autohemagglutinins.

#### CONCLUSIONS

Cold isohemagglutination and autohemagglutination tests were performed on a series of tuberculosis patients. In none of those showing a positive cold isoagglutination reaction was a high titer noted. This suggests that the cold isoagglutination test previously reported<sup>2</sup> may be of value in the differential diagnosis of some forms of early tuberculosis from atypical pneumonia. Since positive reactions were found in low titer in tuberculosis patients as well as in three control patients it should be emphasized that in order that a test be considered positive for atypical pneumonia, the cold agglutinins must be present in high titer.

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# PURPURIC LESIONS IN MENINGOCOCCIC INFECTIONS\*

## DIAGNOSIS FROM SMEARS AND CULTURES OF THE PURPURIC LESIONS

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THE laboratory diagnosis of meningococcus infections has assumed added importance due to the congestion of populations in industrial and camp areas during the war emergency. We have had the opportunity to study a series of 182 cases at the Station Hospital, and the purpose of this report is to record our experiences in culturing the spinal fluid and blood of these patients and to show the value of smears and cultures from the purpuric lesions as an aid in early diagnosis of meningococcus bacteremia.

After considerable experimentation, we developed an efficient culture medium which has enabled us to obtain consistently profuse cultures of the meningococcus after an average incubation period of eighteen hours. In many cases colonies have been observed after only ten hours' incubation. We have also used this medium with remarkable success in the culture of the gonococcus.

### DEXTROSE-STARCH-CHOCOLATE-AGAR FOR CULTURE OF MENINGOCOCCI

14.0 Gm. Bacto dextrose proteose No. 3 agar

15.3 Gm. Bacto nutrient agar

3.2 Gm. soluble starch

Dissolve above in 1,000 c.c. of water and autoclave.  
Bring the medium to a temperature of 90° C. and  
add 5 per cent of rabbit or human blood. Dispense  
sterilely into 6-inch test tubes and slant.

As suggested by Zinsser,<sup>1</sup> we found that the culturing of fairly large quantities of spinal fluid was necessary to obtain satisfactory results. At the same time, it was noticed that organisms from less purulent fluids grew more readily than those from fluids with very high cell counts. Obviously, too many neutrophils carried over on to the medium inhibited the growth to some extent. We therefore made two sets of cultures on purulent fluids. The uncentrifuged spinal fluid was inoculated, one c.c. into a brain heart infusion broth and 0.5 c.c. onto the starch chocolate agar slant, described above. The remaining portion was centrifuged; the supernatant fluid was poured into a tube of broth and a slant was inoculated with the entire sediment. In many cases growth was obtained on both sets of cultures, but in several instances colonies developed only on the medium inoculated with the undimented fluid. Our blood culture

\*From the  
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The report  
by the above aut  
Associated with Fumination  
The clinical report is being written by Captains C. M. Dummer and K. L. Cloninger and will  
be published at an early date

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11, Army Air Forces Training Command, Basic

autopsy material in this series has been written  
under the title "Bilateral Adrenal Hemorrhage  
Septicemia (Waterhouse-Friderichsen Syndrome)."

method has been the inoculation of 100 c.c. of brain heart infusion broth with 10 c.c. of blood, and in a portion of the cases a poured plate was also made, using 3 c.c. of blood to an enriched nutrient agar base. To counteract the sulfa drug, para-aminobenzoic acid, 50 mg. per liter of media, was added.

To supply the carbon dioxide requirements of the meningococcus, Thompson<sup>2</sup> suggests 1 Gm. of sodium bicarbonate and 100 c.c. of 3 per cent sulfuric acid for each 2,500 c.c. volume of the incubation jar. In our work we have used an ordinary mason jar. After placing the culture tubes in the jar, the chemicals are added, and after a few seconds to permit the top gases to escape, the top is clamped on. We did not have the opportunity to try carbon dioxide gas and the Novy or similar jar early in our series. However, it is our belief that the combination of carbon dioxide and moisture is the ideal condition, and we have been reluctant to change the above procedure.

It seems worth while to mention here the extreme variation in the size of the meningococcus as seen on stained smears in this series. Organisms isolated from some cases were in the normal range of 1 micron in diameter, but often the organisms from a case seen on the same day would measure anywhere from 1 to 4 microns in diameter. Size variability in a given culture of meningococcus has been recognized as a characteristic of the organism, but the tremendous size of some organisms isolated from a given case has been a constant surprise to us. A large strain seen on direct smear from the spinal fluid has invariably maintained its size on culture, and a like coordination has been seen in cases of the smaller strains.

We have also noticed a wide variation in colony size in different isolations, the majority measuring 1 mm. to 2 mm. in diameter. However, in some instances the colonies have measured more than twice that size, making it difficult to recognize them as meningococcus colonies. No attempt has been made to coordinate size of organism with size of colony produced.

In the 182 cases studied (Table I), we obtained positive cultures in 167 cases or 91.76 per cent. In four cases, not counted in this series, organisms other than the meningococcus were isolated from the spinal fluid. In two of these cases the pneumococcus was isolated from both the spinal fluid and the blood. In the other two cases, *Micrococcus tetragenus* and streptococcus, gamma type, were found in the spinal fluid, and cultures of the blood were negative.

We have considered all purulent fluids, showing no organisms in the sediment, and negative cultures as probable meningococcus fluids and included these as missed cases from the laboratory standpoint. Fifteen cases (Table I), or a total of 8.24 per cent, belong in this group. These patients were charted as meningitis of undetermined etiology, but coming during a meningococcus epidemic, and due to the fact that other pyogenic organisms with a lesser tendency to autolyze were not found, it seems reasonable to suppose that the meningococcus was probably the causative agent.

A comparison of the culture results in spinal fluid and blood shows much greater success in isolating the organism from the spinal fluid than from the blood, the former being positive in 140 cases while the latter was positive in only 85. We can offer no definite explanations for this, since the two fluids were drawn within a few minutes of each other in practically all cases. However, we do believe that positive blood findings would have been materially increased

if repeat cultures at 24-hour intervals could have been made. This procedure was not followed routinely because of the fact that a high level of sulfadiazine was maintained in these patients. It is interesting to note that percentages of positive blood cultures increased late in the series without change in methods. This brings up the question of modes of invasion and whether or not we have in some cases a meningitis without a meningococcemia.

Twenty cases with a variable rash and a leucocytosis are referred to (Table I). Spinal taps revealed clear, colorless fluids with no increase in cells. Other constituents, such as globulin and sugar, showed no change, and cultures of both the spinal fluid and blood were negative. There is, of course, a possibility that some of these cases were meningococcus septicemias. Zinsser<sup>1</sup> advises a serious consideration of the diagnosis of meningococcus septicemia whenever a prolonged case of fever with leucocytosis and a slight variable rash comes under observation, but due to an absolute lack of laboratory evidence, we have not included them in this discussion.

TABLE I  
SUMMARY OF 182 CASES STUDIED

CASES WITH POSITIVE LABORATORY FINDINGS		
	NO. CASES	PER CENT TOTAL
N. intracellularis		
C.S.F. Pos., Blood Neg.	80	43.96
C.S.F. Pos., Blood Pos.	60	32.97
C.S.F. Neg., Blood Pos.	25	13.73
C.S.F. Neg., Blood Neg., Petechiae Pos.	2	1.10
Total	167	91.76
CASES WITH NEGATIVE LABORATORY FINDINGS		
	NO. CASES	PER CENT TOTAL
Chnically meningitis of undetermined etiology	15	8.24
Total	182	100.00
Cases with purpura and high WBC, but no other positive findings (not included in series)	20	

TABLE II  
CASES WITH CLEAR C.S.F.; POSITIVE ON CULTURE

NO. CASES	CELL COUNT	APPEARANCE	SMEAR	SUGAR	GLOBULIN	CULTURE
10	0-5	Clear	-*	Normal	Normal	+
4	6-10	Clear	-†	Normal	Normal	+
2	11-15	Clear	-†	Normal	Normal	+
4	16-20	Clear	-†	Normal	Normal	+
5	21-50	Clear	-	Normal	Normal	+

\*Four cases in this group showed gram-negative extracellular diplococci in the sediment.

†One case in this group showed gram-negative extracellular diplococci in the sediment.

#### EARLY MENINGITIS CASES WITH CLEAR SPINAL FLUIDS (TABLE II)

Of unusual interest to us has been the number of meningitis cases from which clear, colorless spinal fluids with normal cell counts and normal chemical constituents were obtained. These fluids gave no sediment on centrifugation, and smears made from the small amount of fluid adherent to the walls of the tube after draining, when stained, showed only an occasional leucocyte. In seven cases, gram-negative extracellular diplococci were found, and in two cases, we were able to demonstrate one or two intracellular organisms, but all others resembled perfectly normal fluids. On culture, all 25 of these cases

showed gram-negative diplococci which on typing proved to be a true meningococcus. Elliott<sup>3</sup> reports such a case with a cell count of 11 polymorphonuclear leucocytes, and other cases have been reported, but this group comprising approximately 14 per cent of our total meningococcus cases would seem to indicate one of two things: either the lumbar puncture was done very early, before the cellular response to the infection, or there was a temporary sacculation in parts of the subarachnoid space. In only three of these cases was a later puncture done, and only one of these showed any material elevation in the leucocyte count.

#### THE RAPID DIAGNOSIS OF MENINGOCOCCEMIA FROM SMEARS AND CULTURES OF PURPURIC LESIONS

In 1915, Coles<sup>4</sup> reported the finding of meningococci on smears made from peripheral blood. While counting 2,000 leucocytes, "10 were found to contain typical meningococci." His blood films were stained by Giemsa's method, and his conclusion that the organisms observed were meningococci was based solely on their morphologic similarity to gonococci. In 1916, Netter and Salanier<sup>5</sup> demonstrated the presence of meningococci in the purpuric lesions of two cases of meningococcus bacteremia, and Benda<sup>6</sup> was successful in demonstrating the organisms in sections made from the purpuric lesions. Benda, however, was able to detect them in only one case out of five. Credit must be given to Drigalski<sup>7</sup> for having been the first to demonstrate meningococci in the herpetic vesicles associated with meningococcal infections. In Drigalski's case, a blister-like lesion was apparently produced artificially on the ear lobule by rubbing with green soap and alcohol and making smears and cultures from the extravasated serum. He successfully cultured this material, but failed to obtain viable organisms on subculture. Late in 1916, Netter, Salanier, and Wolfrom<sup>8</sup> found meningococci in smears from purpura in a third case, and in 1917, Netter, Salanier, and Blanchier<sup>9</sup> reported two additional cases. In one, positive smears were obtained, and in another they were also successful in obtaining typical colonies on cultivation. Muir in 1919 reported two cases of meningococcal infection with autopsy findings. In both cases smears from petechiae after death showed gram-negative intracellular diplococci, and in one case he grew meningococci from the petechiae and venous blood after death. Reference for bibliography: Muir, R. J.: Note on the Presence of Meningococci in the Skin Petechiae in Cerebrospinal Fever, *J. Royal Army Med. Corps* **33**: 404, 1919.

Middleton and Duane<sup>10</sup> in 1929 reported an ordinary cover slip smear of peripheral blood revealed a large number of intracellular and extracellular diplococci, resembling meningococci, and in 1931 McLean and Caffey<sup>11</sup> were able to demonstrate meningococci in smears from the purpura in fifteen out of eighteen cases. This report of positive findings in 83 per cent of cases was by far the most important work up to that time, and it now seems surprising that more work was not done in subsequent outbreaks. Nine years later Moss and Schenken<sup>14</sup> observed intracellular diplococci in the granulocytes of ante-mortem blood in one case and demonstrated gram-negative intracellular diplococci in the direct smears made from purpuric spots post-mortem in a second case. In 1942, Kwedar<sup>12</sup> reported that gram-negative intracellular diplococci were found on a blood smear, which were later proved to be meningococci by blood culture.



Monfort and Mehrling<sup>12</sup> stated that microscopically the cutaneous lesions seen in meningococcic bacteremia are shown to be due to direct involvement of the capillaries by the causative organism. Thompkins, in 1943, reported that positive smears were obtained in 39 of 48 cases (about 80 per cent) from purpuric lesions in meningococcic disease. Reference for bibliography: Thompkins, V. N., M. C., A. U. S.: The Diagnostic Value of Smears From Purpuric Lesions of the Skin in Meningococcic Disease, *J. A. M. A.* 123: 31, 1943.

We, like many others, were not appreciative of the tremendous value of the stained smear and culture of blood from the purpuric lesions of meningococcic septicemia until rather late in our series.

#### RESULTS OF SMEARS AND CULTURES FROM PURPURIC LESIONS

Forty cases are recorded in Table III. Blood culture and spinal fluid examination and culture were done simultaneously with the examination of the material from the purpuric lesion.

In Case 1, the patient was extremely ill and already in coma, and because earlier we had failed to diagnose a similar case before death, we decided to attempt cultures and examine stained smears of blood from the petechiae which covered the chest and extremities of the patient. After cleansing the purpuric area with alcohol, we inserted an ordinary 20 gauge needle at an angle almost parallel with the skin surface into the center of the petechial spot, the main concern being not to go deeply enough to draw peripheral blood. By gentle squeezing, several drops of blood and serum were extravasated, and using a sterile platinum loop, cultures and then smears were made of this material. A gram stain was made immediately, and the presence of numerous gram-negative intracellular diplococci was demonstrated (Fig. 1). At approximately eighteen hours, a typical colony growth appeared on the culture medium. On typing, this proved to be a true meningococcus, Type I, and identical with the organism later isolated from both the spinal fluid and blood.

Except for one slight change in technique, we have followed the above procedure in all patients with a petechial or diffuse macular rash. Noting Salanier's reference to the use of a vaccinostyle to scarify the purpuric patch, we have in recent cases, after making the needle puncture, scratched the skin surface over the purpuric area until a slight oozing of blood was obtained. Very light pressure around the area with the fingers produces sufficient material for culture and several smears, with less likelihood of obtaining peripheral blood. We feel that best results have been had with the petechial type of rash, but positive cultures have been obtained from the diffuse type.

Table III (column 1) shows that in 27 cases we were able to find typical gram-negative intracellular diplococci on the smears, thereby permitting a tentative diagnosis within thirty minutes after the patient was admitted to the hospital. In no case in which the organism was found on the direct smear from the purpura did we fail to culture, identify, and type the organism as meningococcus. This, we think, is an important point, since many of the earlier workers did not go beyond finding the organisms on the stained smears. In column two (Table III), it is shown that cultures from the purpuric lesions were positive in 35, or 87.5 per cent, of the total cases. Eight cases in which we did not find typical organ-

isms on the smear were positive on culture. In these cases suspicious extra-cellular bodies resembling single cocci were often seen, but nothing was definite enough to report as diagnostic.

TABLE III  
SMEARS AND CULTURES FROM 40 CASES WITH PURPURIC LESIONS

	SMEARS OF PURPURA	CULTURES OF PURPURA	OTHER LABORATORY FINDINGS	
			C.S.F. CULTURE	BLOOD CULTURE
Case 1	+	+	-	+
Case 2	-	-	+	-
Case 3	+	+	+	-
Case 4	+	+	-	-
Case 5	-	+	-	-
Case 6	-	-	+	+
Case 7	-	+	+	-
Case 8	-*	+	+	+
Case 9	-	-	+	-
Case 10	-	-	+	-
Case 11	+	+	-	+
Case 12	+	+	-	+
Case 13	+	+	+	+
Case 14	+	+	+	+
Case 15	+	+	+	+
Case 16	+	+	+	+
Case 17	+	+	+	+
Case 18	+	+	+	+
Case 19	+	+	+	+
Case 20	-	+	+	+
Case 21	+	+	+	+
Case 22	+	+	+	+
Case 23	+	+	+	+
Case 24†	+	+	+	+
Case 25	-	+	-	+
Case 26	+	+	+	+
Case 27	+	+	+	-
Case 28	+	+	-	+
Case 29	+	+	+	+
Case 30	+	+	+	+
Case 31	+	+	+	+
Case 32	-	+	+	+
Case 33	-	+	+	+
Case 34	-	-	+	-
Case 35	+	+	+	+
Case 36	+	+	+	+
Case 37	+	+	+	-
Case 38	+	+	-	+
Case 39	+	+	+	+
Case 40	-	+	+	+
Per Cent Pos.	67.5	87.5	82.2	75

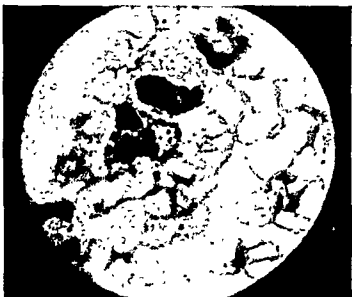
\*Slides in this case suspicious but not diagnostic.

†Meningococci were isolated from aspirated knee fluid in this case.

We have isolated the meningococcus from purpura in four additional cases since this paper was written.

The experience of Coles,<sup>4</sup> Middleton,<sup>10</sup> and Kwedar<sup>12</sup> in finding the organisms in ordinary smears of the peripheral blood should be kept in mind, but their cases were unquestionably overwhelming infections, and our experience has not shown it to be a dependable method of diagnosis. In several cases after finding meningococci in smears from the purpuric lesions, we have diligently searched for organisms on ordinary smears of the peripheral blood, but without success.

A comparative analysis of complete findings, including spinal fluid and blood cultures, shows that in 40 cases the cultures of the lesions were positive in 35, while the blood cultures were positive in only 30 cases. It is interesting



A.



D.



B.



E.



C.

...  
 X1200. D shows a cluster of extra-  
 cellular meningococci (Case 36, Table  
 III). The erythrocytes have been de-  
 stroyed by fixing. (Magnification  
 X1200.) E shows two pus cells with  
 large numbers of meningococci (Case  
 37, Table III). Wright's stain; mag-  
 nification X600.

to note that the percentage of positive findings in the cultures of the purpura is higher than the cultures of either the blood or spinal fluid. However, in arriving at comparative values of diagnostic methods, it is important to remember that many cases of meningococcic septicemia with purpura do not develop into spinal meningitis, and in these cases the evidence presented weighs heavily in favor of smears and culture of material from the purpuric lesions (87.5 per cent positive purpura and 75 per cent positive blood cultures). Table III also shows two cases, 4 and 5, in which the material from the purpuric areas furnished the only positive diagnosis in the case. In Case 4, typical gram-negative intracellular diplococci were found on the smear, and positive cultures were obtained. Diligent search in Case 5 failed to demonstrate organisms on the smears, but cultures showed typical colonies which on typing proved to be meningococci. Cultures of the venous blood and spinal fluid done simultaneously, and as in all other cases prior to initiation of the treatment, were both negative. The technique of making and examining the smears is such that we feel it should in all cases be done by the bacteriologist or pathologist and not entrusted to a laboratory technician.

TABLE IV  
TYPES OF MENINGOCOCCI

	NO. CASES C.S.F. OR BLOOD	PER CENT TOTAL	NO. CASES PETECHIAE	PER CENT TOTAL
Type I	153	91.6	34	97.0
Type II	7	4.3	0	0
Type IIa	2	1.2	0	0
Type IV	1	0.6	1	2.9
Did Not Type	4	2.4	0	0
Total Cases	167		35	

TABLE V  
SUMMARY OF DEATHS AND MORTALITY RATE

Number of proved meningococcic meningitis and meningococcemia cases	167
Number of deaths in this group	4
Mortality rate (per cent)	2.39
Total number of cases, including 15 of undetermined etiology	182
Total number of deaths	6
Mortality rate (per cent)	3.29

Approximately 75 per cent of meningococcus infections in this hospital have shown some degree of purpura; therefore it seems very likely that an earlier initiation of our present method of examining smears and culturing the extravasated blood and serum from these areas would have afforded a diagnosis in some of the 20 cases charted as purpura of undetermined etiology (Table I).

#### SPECIFIC TYPES OF MENINGOCOCCI

In Table IV, we have recorded the specific types of meningococci found in our series. It is significant that almost all of the cases are classed as Type I. In 153 cases or 91.6 per cent of the total, the organism isolated from the blood, spinal fluid, or both, proved to be Type I meningococcus. In 4 cases, or 2.4 per cent, we did not type the organisms. Three of these cultures were identified by agglutination with a polyvalent meningococcus horse serum, and we did not

at that time have the antisera to do specific typings. In the fourth case, the organisms agglutinated when tested with the polyvalent serum, but we were unable to obtain a specific type. Of the 35 meningococcus cultures isolated from purpuric lesions, 34 or 97 per cent were Type I.

Cultures of the nasopharynx were done on 3,846 meningitis contacts. The carrier rate averaged from 16.5 per cent to 23 per cent.

In Table V, a summary of deaths in both proved and unproved cases is given. In the 167 diagnosed cases there were only 4 deaths. Two other deaths occurred among the cases classed as of unknown etiology.

#### SUMMARY

1. Meningococci were demonstrated in smears from the purpuric lesions in 27 or 67.5 per cent of 40 cases of meningococcic infections. On culture, the organisms were isolated in 35 or 87.5 per cent. In two cases, this offered the only bacteriologic diagnosis.

2. Positive spinal fluid cultures were obtained in 25 cases of meningitis showing clear fluids with normal chemical constituents.

3. A culture medium for the isolation of the meningococcus is described.

#### CONCLUSIONS

Smears and cultures from purpuric lesions in meningococcemia offer a rapid method of diagnosis. Cultures of the purpuric areas proved to be the most satisfactory diagnostic procedure.

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# PLASMA CLOT TENSILE STRENGTH

## II. THE EFFECT OF SOME PHYSICAL FACTORS, ANTICOAGULANTS AND COAGULANTS\*

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THE use of autologous plasma clots for the suture of peripheral nerves has raised many questions concerning the factors which may influence their physical characteristics.<sup>1, 2</sup> In earlier reports,<sup>3, 4</sup> a simple method for measuring the tensile strength<sup>2</sup> of plasma clots was described, and the tensile strength was shown to be roughly proportional to the fibrinogen content of the plasma from which they were formed. This report concerns the results of experiments designed to evaluate the influence of other physical and chemical factors on the tensile strength of plasma clots.

### MATERIALS AND METHODS

Human venous blood was used in these experiments. It was withdrawn into a syringe coated with mineral oil. Those samples which were used to prepare unmodified plasma (obtained from blood to which no anticoagulant is added) were transferred to paraffin-lined test tubes packed in ice and then centrifuged in 250 c.c. metal cups filled with ice. Those portions of blood which were used for studies of plasma prepared with anticoagulants were placed in tubes containing measured amounts of the anticoagulant. After centrifuging at approximately 2500 revolutions per minute for five minutes, the plasma was pipetted into test tubes of approximately 8 mm. internal diameter (50 sq. mm. cross section) and allowed to clot either spontaneously or upon the addition of the specified reagents. The tests of tensile strength were carried out on clots which were allowed to stand for a minimum time interval of one hour in the water bath after complete coagulation had occurred. Tensile strength determinations were done in at least triplicate on the clots prepared from each sample of plasma.

The technique used for measuring the tensile strength of plasma clots was essentially that previously described.<sup>3</sup> Careful examination of the results of measurements of the tensile strength of numerous clots prepared from the same plasma indicated that the scatter was so large that approximately 30 clots were necessary to establish a significant mean value. In an attempt to obtain closer agreement between repeated determinations on clots prepared from the same

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†The term tensile strength as used in this paper refers merely to the load at which the clot breaks under the specified conditions of preparation and testing.

material, the procedure followed in preparing and testing the clots was systematically modified to eliminate various suspected sources of error. Since the tensile strength of plasma clots was found to increase when more fluid was expressed, this effect was standardized by transferring the clot to a watch glass, where the excess fluid was allowed to drain off. The clot was touched repeatedly against clean filter paper until a partly dry spot remained. It was then suspended between the jaws of a rubber-sleeved Allis clamp fixed to a rigid support. Injury by rough handling in changing weights was diminished by suspending a paper cup by a silk thread from the lower clip attached to the clot and slowly running in water from a burette with an S-shaped delivery tube. The tensile strength of the clot was taken as the sum of the weights of the cup and clip (10 grams) and the volume of water in cubic centimeters at which the clot broke. Tugging or torsion on the clot during the addition of weights was further reduced by slightly deflecting the string from the lower clamp to the paper cup over a ball-bearing pulley so that the occasional twisting of the cup was not transmitted. Since the range of variation in the results was not decreased by these apparent improvements in technique, the variability was considered to be a property of the clots themselves rather than an artifact of the method of testing. The modifications in the technique of measurement, however, were retained because of their convenience. Since averages of groups of 30 or more clots were necessary to obtain significant mean values, it was not practicable to obtain large enough samples of blood from individual subjects to provide sufficient plasma to permit the performance of each complete experiment on a single specimen. It was therefore necessary to have a method of calculation which would avoid undue weighting of the average by determinations on samples of plasma yielding exceptionally strong clots. This was achieved by reducing the absolute values of the tensile strength of plasma clots to relative units in a scale on which the average tensile strength of the unmodified clots from each plasma was fixed as 100. Such a relative tensile strength (RTS) was obtained by dividing 100 times the absolute tensile strength of each clot by the average tensile strength of the unmodified plasma clots prepared from the same blood specimen. In tabulating the results of each experiment, the mean RTS was calculated for all the clots which were prepared and tested under the same conditions. The standard deviation and standard error were computed in the manner suggested by Pearl.<sup>5</sup>

#### EXPERIMENTS

##### *I. The Effect of the Physical Conditions of Coagulation on the Tensile Strength of Plasma Clots.—*

###### *A. Size of Tube.*

Although the factors and mechanisms determining the tensile strength of plasma clots are not entirely understood, an elementary analysis of the physical system involved suggests at once that the tensile strength should be proportional to the internal cross-sectional area of the tube in which the clot is formed. This phenomenon, assumed without proof by Kristenson,<sup>6</sup> was studied by comparing series of unmodified clots prepared in tubes of three different diameters. For each specimen of plasma, the average strength of the clots formed in tubes of

8 mm. diameter was used as a basis for calculating the RTS of the clots formed from the same plasma in tubes of different diameter. The results of these experiments are shown in Table I, from which it is clear that the mean RTS per square mm. of clot is constant within limits of experimental error when the area of the tube varies through a range of 300 per cent.

### B. Temperature.

To determine whether variation in temperature during incubation and measurement could account for some of the observed variations in the tensile strength of clots prepared from the same sample of plasma, a series of clots were prepared and measured in a constant temperature room at 37° C. For this purpose a single specimen of sodium citrate plasma was used, and the clots formed by the addition of dry calcium chloride. All glassware, reagents, and plasma were brought to 37° C. before coagulation was begun and kept at that temperature throughout the entire experiment. The tensile strength of these clots showed the same amount of variation as clots prepared and tested by the usual method.

TABLE I

THE EFFECT OF THE CROSS-SECTIONAL AREA OF THE TUBE IN WHICH PLASMA CLOTS ARE FORMED ON THEIR TENSILE STRENGTH

NUMBER OF CLOTS TESTED	INTERNAL DIAMETER OF TUBE	CROSS-SECTIONAL AREA OF TUBE	MEAN RTS	MEAN RTS PER SQUARE MILLIMETER	STANDARD ERROR OF MEAN RTS PER SQUARE MILLIMETER
39	8.0 mm.	50 mm. <sup>2</sup>	100	2.00	0.07
37	10.5 mm.	87 mm. <sup>2</sup>	192	2.21	0.11
29	14.0 mm.	154 mm. <sup>2</sup>	284	1.84	0.11

TABLE II

THE EFFECT OF THE TEMPERATURE AT WHICH PLASMA CLOTS ARE FORMED ON THEIR TENSILE STRENGTH

NUMBER OF CLOTS TESTED	TEMPERATURE ° C.	RTS	STANDARD ERROR OF RTS
33	37	100	3.1
34	20	86	3.8
63	37	100	3.2
62	10	67	3.2

To study the effect of slowing coagulation by lowering the temperature, a series of experiments was carried out on unmodified plasma. The RTS of clots formed at 10° C. and 20° C. was computed by taking the average tensile strength of clots formed at 37° C. as 100. At the lower temperatures, the rate of coagulation was greatly retarded and was found to vary markedly from tube to tube. At 37° C. coagulation was usually complete in 20 to 30 minutes, at 20° C. it usually required 2 to 3 hours and at 10° C., 20 to 24 hours. In general, the clots were allowed to stand for several hours after coagulation appeared complete, but because of the uncertainty as to whether the process was complete, the expressed serum was incubated at 37° C. and the determination discarded if further coagulation occurred. The results of these experiments shown in Table II indicate a definite reduction in RTS when the clots are slowly formed at the lower temperatures.



### C. Nature of Surface of Tube in Which Coagulation Occurs.

It is well known that the beginning of coagulation in shed blood is markedly delayed when the blood is collected in a paraffin-lined vessel,<sup>7</sup> and this phenomenon has been applied in the preparation of unmodified plasma clots for nerve suture<sup>1, 2</sup> and tensile strength studies.<sup>3, 4</sup> To evaluate the effect of the surface of tube in which the clots are formed on their tensile strength, a series of unmodified plasma clots was prepared in tubes whose inner surfaces were coated with thin layers of paraffin, latex, and colloidal graphite (aquadag).

It was noted in these experiments as in most of the other studies on unmodified plasma, that the coagulation time varied greatly from clot to clot in each series prepared under the same conditions from the same plasma. In general, however, the clots in rubber or latex-lined tubes formed somewhat more slowly and those in the aquadag-lined tubes more rapidly than those prepared in plain glass tubes. The relative tensile strength of these clots was computed by taking the average value of the tensile strength of the clots formed from each plasma in plain glass tubes as 100.

The results of these experiments are shown in Table III. It is clear that within the range of experimental error, the nature of the surface of the tubes in which the clots were formed had little influence on their tensile strength.

TABLE III

THE EFFECT OF THE LINING OF THE TUBE IN WHICH PLASMA CLOTS ARE FORMED UPON THEIR TENSILE STRENGTH

NUMBER OF CLOTS TESTED	LINING OF TUBE	MEAN RTS	STANDARD ERROR
50	Glass	100	2.8
40	Latex	117	6.1
32	Paraffin	102	6.9
32	Graphite (aquadag)	84	6.2

### D. Time.

The effect of the period of incubation upon the tensile strength of plasma clots may be important in relation to their suitability as nerve suture material, since if the clots rapidly deteriorate before fibrous tissue replacement has occurred, then the nerve ends may separate. In an earlier report, it was concluded that unmodified plasma clots formed in glass tubes reach their maximum strength about twenty minutes after the beginning of coagulation. In these experiments the beginning of coagulation was determined by tipping the tube at short intervals, noting the time when the plasma failed to flow (tip test). Further study has shown that if the tubes are not agitated, and the end point of coagulation is determined by the appearance of turbidity, there is much variation in the coagulation time among different samples of the same plasma and also in the time required for complete coagulation. To investigate this phenomenon further, 22 one c.c. samples of the same chilled unmodified plasma were placed in a water bath at 37° C. At short intervals these tubes were inspected without agitation, and those in which coagulation appeared to be complete as judged by the attainment of maximum turbidity were removed and tested for tensile strength. It was found that among the tubes in which opacity appeared to be maximal, most of the clots which were examined within the first

forty minutes showed evidence of incomplete coagulation either by sacklike form of the clots or by the spontaneous coagulation of the expressed fluid. Among those clots which did not show the signs of incomplete coagulation, no significant difference in the tensile strength was observed between those clots incubated twenty minutes and those incubated for longer intervals up to one hundred and forty minutes.

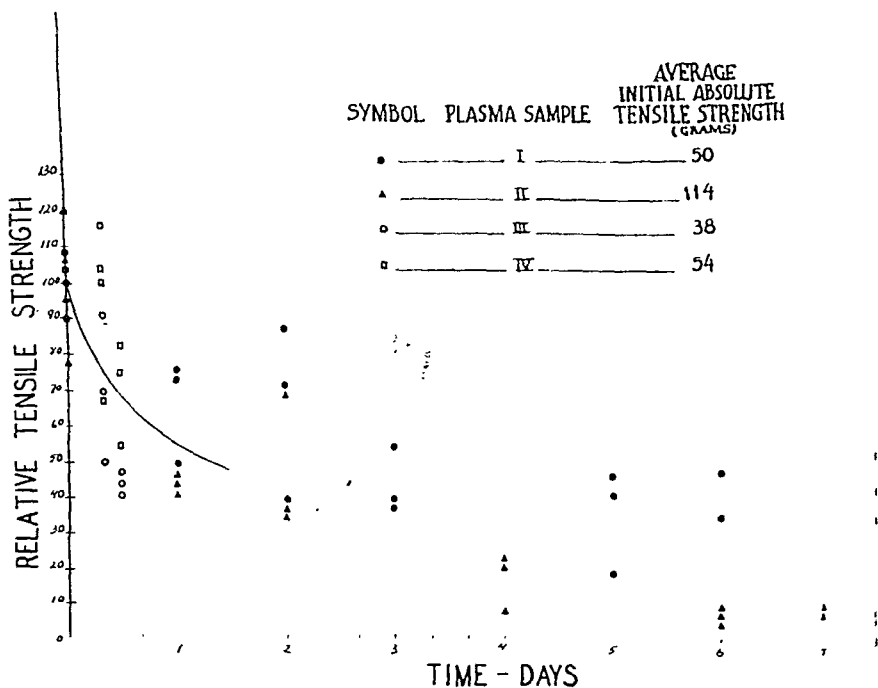


FIG. 1 - CHANGE IN TENSILE STRENGTH OF PLASMA CLOTS WITH TIME

To study the change in tensile strength of plasma clots on longer scale it was necessary to take precautions against bacterial contamination and the loss of water and dissolved gases by evaporation from the surface. For this, clots were prepared from unmodified plasma obtained under aseptic conditions and placed in sterile test tubes into which a sterile cotton plug soaked in melted paraffin was pushed to within about 1 cm. of the surface of the clot. The tubes were then closed with sterile corks and sealed with paraffin. On two batches of plasma handled in this way and incubated at 37° C., triplicate tensile strength determinations were done at the beginning of the experiment and at various time intervals thereafter. The RTS was calculated using the initial tensile strength for the clots from each plasma at the beginning of the experiment as 100. The results are shown graphically in Fig. 1. In spite of the scatter of individual determinations, it is clear that the tensile strength declines rapidly within the first 24 hours reaching a value 50 to 60 per cent of the initial strength and then falling more slowly to about 20 per cent of the initial value at the end of eight days. Partial lysis was noted in some of the clots at the end of the fourth day.

## II. *The Effect of Tissue Extracts on the Tensile Strength of Plasma Clots.*—

Extracts of mammalian tissue contain substances, probably lipoprotein in nature, which accelerate the coagulation of fresh blood or plasma. The exact mechanism of their action is uncertain, but it is known that in the presence of calcium ions, they accelerate the conversion of prothrombin to thrombin which in turn catalyzes the conversion of fibrinogen to fibrin.<sup>7</sup>

Since lung and muscle extract have been added to the unmodified plasma used in some of the studies on plasma clot suture,<sup>2</sup> the effect of these substances on the strength of clots was investigated. Tissue extracts were prepared by thoroughly grinding fresh human, dog, or rabbit lung or muscle in a mortar with clean sand and centrifuging the mixture. The supernatant fluid then was removed and used undiluted, or diluted five and ten times with Ringer's or normal saline solution. In testing these preparations, one drop was added to one c.c. of chilled unmodified plasma. The results of the tensile strength determinations are shown in Table IV.

TABLE IV

THE EFFECT OF MUSCLE AND LUNG EXTRACT ON THE RELATIVE TENSILE STRENGTH OF PLASMA CLOTS\*

TYPE OF PLASMA	COAGULATING AGENT	NUMBER OF SAMPLES OF PLASMA	NUMBER OF CLOTS TESTED	MEAN RTS	STANDARD ERROR
Unmodified	None	12	36	100	2.5
Unmodified	Muscle extract	12	51	72	4.8
Unmodified	Muscle extract diluted 1:5	12	51	83	3.6
Unmodified	Muscle extract diluted 1:10	12	41	87	4.9
Unmodified	Lung extract	12	36	34	3.9
Unmodified	Lung extract diluted 1:5	12	36	66	3.9
Unmodified	Lung extract diluted 1:10	12	29	78	6.1

\*The data presented in the above table are not strictly comparable to those presented in Tables V and VI, since but one drop of muscle or lung extract was added to the unmodified plasma. Hence the dilution factor in these experiments with muscle and lung extract is negligible.

The muscle and lung extracts were diluted with either Ringer's or normal saline solution.

The coagulation accelerating tissue extracts all tended to weaken the clots produced from unmodified plasma, an effect more marked with lung extract, which produced more acceleration of coagulation than muscle extract. That the effect is not due entirely to the increased coagulation rate is indicated by the finding that the diluted extracts, which had about the same effect on the rate of coagulation, weakened the clots less than the more concentrated ones.

## III. *The Effect of Anticoagulants on the Tensile Strength of Plasma Clots.*—

### A. Decalcifying Anticoagulants.

The most commonly used anticoagulants are the soluble salts of citric and oxalic acid which prevent coagulation by removing calcium ions from solution. Plasma prepared by centrifuging blood treated with these anticoagulants may be re-coagulated by the addition of suitable amounts of soluble ionized calcium salts.

Solutions isotonic with blood plasma were used, since other concentrations would produce a shift of water between the red blood cells and the plasma until equilibrium was re-established.

### 1. Sodium Citrate.

A 3.8 per cent solution of hydrated sodium citrate is isotonic with blood plasma. Preliminary trials of several different blood samples containing varying amounts of sodium citrate indicated that one part of citrate to 19 parts of whole blood is a safe anticoagulant for most specimens and that one part to 14 of whole blood is safe for all bloods examined. These figures, representing 0.14 per cent to 0.18 per cent anhydrous sodium citrate in the final mixture, are in good agreement with those (0.15 per cent to 0.20 per cent) reported as minimal anticoagulant concentrations in the literature.<sup>8</sup>

The minimal amount of calcium chloride necessary to coagulate citrated plasma may be calculated from the theoretical studies of Ransmeier and McLean,<sup>9</sup> but since more than minimal amounts are probably optimal, the data of Nygaard<sup>10</sup> were used as a starting point. A rough calculation from these data suggests that 0.1 c.c. of a 0.5 per cent to 2.5 per cent solution of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  should act as a convenient and effective coagulant.

Citrated plasma was prepared from the blood of each of two healthy normal adults using one part of a 3.8 per cent sodium citrate solution to 19 parts of blood. Nine-tenths c.c. portion of this plasma was mixed in each tube with 0.1 c.c. of a solution of calcium chloride of 0.5, 1.0, 1.5, 2.0, or 2.5 per cent. At least three clots were prepared at each calcium chloride concentration. The tensile strength was determined on these clots and on unmodified clots prepared from each sample of plasma. The RTS was calculated. As was expected from Nygaard's observations, no significant difference was noted between the clots prepared with the different amounts of calcium chloride.

A similar experiment was carried out using a fixed amount of calcium chloride and varying the concentration of the sodium citrate solution from 5 volumes to 11 volumes per cent. Here the larger concentration of citrate seemed to give somewhat weaker clots, but the difference was not large compared with the difference between triplicates of the same sample.

On the basis of these two experiments, it was considered that slight differences in citrate concentration such as might occur as a result of unusually high or low hematocrit values would be unlikely to cause significant differences in plasma clot tensile strength, and that small differences in the proportion of calcium chloride to sodium citrate would also have relatively little effect.

### 2. Potassium and Ammonium Oxalate.

A 1.6 per cent solution of a mixture of 4 parts of dry potassium oxalate and 6 parts of dry ammonium oxalate is isotonic with blood plasma. Preliminary trials of this solution with different proportions of whole blood indicated that one part of the oxalate solution is a safe anticoagulant for 19 parts of blood. Calculations show that this figure corresponds closely to Scott and Chamberlain's<sup>11</sup> value which represents four times the amount necessary to combine with the plasma calcium. Experiments with oxalate solution similar to those carried out with the citrate solution gave analogous results. When, however, 1 part of oxalate solution was added to 9 parts of blood, clotting occurred although the clots were very weak.

To study in detail the RTS of clots prepared from recalcified plasma, unmodified, citrated, and oxalated plasmas were prepared from the blood of each of ten subjects. Isotonic potassium and ammonium oxalate solution was used in the proportion of one part to 19 parts of blood. Isotonic sodium citrate solution was used in the proportions of one part to 14 parts and one part to 19 parts of blood. One-tenth c.c. of 2 per cent calcium chloride solution was added to 0.9 c.c. of chilled plasma containing the anticoagulant and mixed by inverting the test tube three times against a clean cork. The RTS of the resulting clots were determined.

To evaluate the effect of the dilution of the plasma by the added reagents, a series of determinations of RTS was done on additional groups of clots prepared from unmodified plasma to which isotonic sodium chloride solution was added in the proportion of 10 per cent and 20 per cent of the final volume. As a further control, the RTS was determined on a series of clots prepared from unmodified plasma to which the citrate and oxalate salts had been added in dried form in the same proportions in which they would occur in plasma obtained from blood to which one part of isotonic anticoagulant solution had been added for each 14 parts of blood. These plasmas were coagulated by adding 1 c.c. portions to test tubes containing the calcium chloride dried from 0.1 c.c. of a 2 per cent solution. The results of all the studies on decalcifying anticoagulants and dilution controls are shown in Table V.

When an isotonic solution of an anticoagulant salt is added to whole blood, the plasma is diluted by it. It may be easily calculated\* that clots prepared from 0.9 c.c. of such plasma and 0.1 c.c. of calcium chloride solution contain fibrinogen, and other solids present in only about 0.8 c.c. of original blood plasma and their mean RTS may, therefore, be expected to be about 20 per cent less than that of clots prepared from unmodified plasma.<sup>4</sup> In Table V it is seen that the clots prepared from unmodified plasma diluted with 20 per cent of saline solution averaged 27 per cent weaker, while the citrated clots were 21 per cent weaker, and the oxalated clots 30 per cent weaker than clots prepared from unmodified plasma. The standard errors of these results are large enough so that it cannot be concluded that any of these values is significantly different from the 20 per cent weakening effect expected as the result of dilution alone.

For citrated plasma clots, this interpretation is further strengthened by the finding that unmodified plasma stabilized by the addition of dry sodium citrate and coagulated by the addition of dry calcium chloride gave clots whose mean RTS was not significantly different from 100, the mean RTS of the unmodified clots.

On the other hand, the clots prepared from unmodified plasma treated with dry potassium ammonium oxalate and dry calcium chloride had a mean RTS 14 per cent less than that of the unmodified clots. Since the standard

\*The volume of original plasma in the final 1 c.c. clot is  $\frac{0.9 \text{ c.c.} \times \text{volume of plasma}}{\text{volume of plasma} + \text{volume of anticoagulant}}$   
 The volume of plasma equals volume of blood  $\times$  (1 - hematocrit). With a hematocrit of 0.45:  
 1 c.c. of anticoagulant to 14 c.c. of blood gives:  $\frac{0.9 \text{ c.c.} \times (14 \times 0.55)}{(14 \times 0.55) + 1} = 0.80 \text{ c.c.}$   
 1 c.c. of anticoagulant to 19 c.c. of blood gives:  $\frac{0.9 \text{ c.c.} \times (19 \times 0.55)}{(19 \times 0.55) + 1} = 0.82 \text{ c.c.}$

error is 5.1 per cent, this may be considered as representing a small but definite weakening effect due to the oxalate, possibly caused by the presence of crystals of calcium oxalate among the fibers of the clot.

### B. The Use of Heparin as an Anticoagulant.

The anticoagulant action of heparin has recently been reviewed by Quick,<sup>7</sup> who summarizes as follows: "first, it prevents the liberation of thromboplastin from platelets; second, it prevents, with the aid of a plasma co-factor, the conversion of prothrombin into thrombin; and third, it forms with serum albumin a strong antithrombin." Thus, heparin, by acting as an inhibitor of coagulation at three successive stages of the clotting process, is admirably suited for the role for which it has often been suggested, but never conclusively proved—that of the normal physiologic anticoagulant.<sup>7, 12</sup> For our purposes, it has the added advantage of being extremely potent in small amounts.

TABLE V

THE EFFECT OF RECALCIFICATION OF CITRATED AND OXALATED PLASMA AND OF DILUTION WITH SALINE SOLUTION ON THE RELATIVE TENSILE STRENGTH OF CLOTS

TYPE OF PLASMA	RECALCIFIED OR DILUTED WITH	NUMBER OF SAMPLES OF PLASMA	NUMBER OF CLOTS TESTED	MEAN RTS	STANDARD ERROR
Unmodified	Nothing	10	46	100	2.8
Sodium citrate (1:20)	0.1 c.c. of 2 per cent calcium chloride	8	31	73	5.6
Sodium citrate (1:15)	0.1 c.c. of 2 per cent calcium chloride	10	39	83	6.6
Sodium citrate (1:15 or 1:20)	0.1 c.c. of 2 per cent calcium chloride	10	70	79	4.3
Ammonium and potas- sium oxalate solution (1:20)	0.1 c.c. of 2 per cent calcium chloride	10	40	70	4.3
Unmodified	Nothing	16	87	100	2.9
Unmodified	0.1 c.c. of isotonic sodium chloride	11	66	89	4.4
Unmodified	0.2 c.c. of isotonic sodium chloride	13	79	73	3.3
Unmodified	Nothing	5	35	100	4.4
Unmodified dry sodium citrate	2 mg. dry calcium chloride	5	40	97	5.3
Unmodified dry am- monium and potassium oxalate	2 mg. dry calcium chloride	5	38	86	5.1

Preliminary tests of a 1 per cent solution of the sodium salt of heparin (Liquaemin: Roche) indicated that while 0.01 mg./c.c. of blood is adequate to prevent coagulation of some specimens, 0.05 mg./c.c. is necessary for a safe anti-coagulant effect, and this amount was used in all subsequent experiments.

To coagulate heparinized plasma, one must either supply an excess of thrombin, or else neutralize the heparin so that the normal coagulation mechanism can operate. Both methods were studied.

### 1. Coagulation of Heparinized Plasma by the Addition of Rabbit "Clotting Globulin."

"Clotting Globulin" (Lederle)\* is derived from rabbit plasma, and it has been demonstrated to have the properties of thrombin.<sup>13, 14</sup> It is supplied in a 10 per cent solution which contains a preservative.

\*Kindly supplied by the Lederle Laboratories.

In exploratory experiments with varying amounts of globulin as the coagulating agent for heparinized plasma, it was found that 0.1 c.c. of the stock 10 per cent solution added to 0.9 c.c. of heparinized plasma or 0.05 c.c. of the solution mixed with 0.95 c.c. of plasma gave clots which were up to 50 per cent weaker than the average clot prepared from unmodified plasma. One-tenth c.c. of a 2 per cent solution to 0.9 c.c. of plasma yielded clots from 20 per cent to 80 per cent weaker than unmodified plasma clots, and 0.05 c.c. of 2 per cent solution added to 0.95 c.c. of plasma resulted in clots which were so weak and friable that their strength could not be measured.

Unmodified plasma and heparinized plasma (0.05 c.c. of 1 per cent solution of liquaemin to 10 c.c. of blood) were prepared from blood taken from each of ten patients. Nine-tenths c.c. of heparinized plasma was coagulated with 0.05 c.c. or 0.1 c.c. of 10 per cent "Clotting Globulin" solution. Unmodified clots were prepared as usual. The tensile strengths were measured and the RTS calculated. The results are shown in Table VI.

TABLE VI

THE EFFECT OF RABBIT "CLOTTING GLOBULIN" AND PROTAMINE SULFATE ON THE RELATIVE TENSILE STRENGTH OF HEPARINIZED PLASMA CLOTS\*

TYPE OF PLASMA	COAGULATING AGENT	NUMBER OF SAMPLES OF PLASMA	NUMBER OF CLOTS TESTED	MEAN RTS	STANDARD ERROR
Unmodified	None	10	46	100	2.8
Heparinized	"Clotting Globulin" 0.05 c.c.	3	12	60	12.6
Heparinized	"Clotting Globulin" 0.1 c.c.	7	28	37	6.2
Heparinized	"Clotting Globulin" 0.05 c.c. or 0.1 c.c.	10	40	58	6.3
Unmodified	None	10	54	100	4.0
Heparinized	Protamine sulfate 0.1 mg.	10	41	100	3.1
Heparinized	Protamine sulfate 0.125 mg.	10	41	100	6.0
Heparinized	Protamine sulfate 0.15 mg.	5	20	102	6.0
Heparinized	Protamine sulfate 0.1, 0.125 or 0.15 mg.	10	102	101	3.0

\*The final volume of all clots was 1 c.c.

## 2. Coagulation of Heparinized Plasma by Means of Protamine Sulfate.

In attempts to obtain an anticoagulant with prolonged action, Chargaff and Olson<sup>15</sup> precipitated heparin with protamine sulfate but found that its effect was completely neutralized. In further studies they showed that protamine sulfate is capable of neutralizing the anticoagulant effect of heparin both in vitro and in vivo. Protamine sulfate, therefore, seemed a suitable reagent for removing heparin to permit coagulation to occur in a normal way. (Protamine sulfate itself is a weak inhibitor of coagulation<sup>15</sup> rather than a coagulant.)

To determine optimal conditions, varying amounts of protamine sulfate were added in 0.1 c.c. of water to 0.9 c.c. of heparinized plasma in a water bath at 37° C., and coagulation or its absence was noted at intervals. It was found that with 0.09 mg. of protamine sulfate or less, coagulation was markedly delayed or prevented, while with 0.1 mg. or more, coagulation was about as rapid as in unmodified blood.

Unmodified plasma and heparinized plasma were prepared from blood drawn from each of ten patients. Nine-tenths c.c. sample of heparinized plasma was coagulated in quadruplicate with 0.1 c.c. of aqueous solutions con-

taining 0.10, 0.12, and 0.15 mg. of protamine sulfate respectively. The RTS of the clots were determined (Table VI).

The volume and molar concentration of heparin used as an anticoagulant in our experiment was so small that it introduced no significant dilution, but the added coagulating agents produced a slight but definite dilution—5 per cent in the experiment with 0.05 c.c. of "Globulin" solution, 10 per cent in all other experiments. The clots prepared from heparinized plasma coagulated with rabbit "Globulin" had a mean RTS about 40 per cent less than that of the clots prepared from unmodified plasma and must be considered significantly weaker than the latter.

On the other hand, the clots prepared by neutralizing the heparin with protamine sulfate had a mean RTS which was the same as that of the unmodified clots and slightly but significantly higher than the mean RTS of the clots prepared from unmodified plasma diluted with a corresponding volume of saline.

#### DISCUSSION

Some of the implications of the results of our studies concerning the influence of various factors on the tensile strength of plasma clots are clear. As would be expected, the mean relative tensile strength is proportional to the cross-sectional area of the tube in which the clot is formed, and inversely proportional to the amount of dilution of the plasma by saline solution. These findings are consistent with the previous observation<sup>4</sup> that clots of the same size prepared from different specimens of plasma have tensile strengths roughly proportional to their fibrinogen concentration.

The mean RTS of plasma clots is slightly decreased by the addition of small amounts of muscle extract, and by lowering the temperature from 37° C. to 20° C., while it is slightly increased in latex-lined tubes as compared with glass tubes. Insofar as these compensating factors are concerned, it is likely that the tensile strength of plasma clots *in vivo* is similar to that observed *in vitro*. It is significant, however, that the clots *in vitro* show a definite weakening on standing even when contamination, and evaporation of dissolved gases is prevented.

Some conclusions may be drawn as to the suitability of various types of anticoagulants and coagulants for the preparation of plasma clots for surgical use. The findings on the mean RTS of clots prepared by the recalcification of citrated plasma are consistent with the concept that sodium citrate solution in suitable concentration exercises no effect on the RTS of plasma clots other than that of simple dilution. Since it has been shown that within a fairly wide range the RTS is independent of the volume of citrate solution used as the anticoagulant and the amount of calcium chloride used to coagulate the plasma, and since sterile sodium citrate solution is so readily available wherever surgery is performed, citrated plasma clots would seem to be worthy of further investigation.

The clots prepared by the recalcification of oxalated plasma have a mean RTS slightly but definitely lower than would be predicted as a result of simple dilution. Although this effect is small as compared to the differences between some of the individual clots in the series, the presence of calcium oxalate crystals in the clots and the absence of any theoretical or practical advantage of oxalate over citrate suggest that oxalated plasma is an unsuitable source of clots.



When heparin is used as the anticoagulant, the plasma clots prepared by the addition of the thrombin-like substance from the globulin fraction of rabbit plasma had a mean RTS definitely lower than that expected as a result of dilution alone. Moreover, the fact that they contain relatively large amounts of rabbit protein might make such clots undesirable.

When protamine sulfate solution is used to inactivate the heparin in heparinized plasma and permit the normal coagulation mechanism to operate, the resulting plasma clots have a mean RTS approximately equal to that of unmodified plasma. Since the volume and molar concentration of the heparin-protamine sulfate solutions are so low, the heparin-protamine plasma clots may be considered chemically most similar to unmodified clots and are certainly well worthy of further study in connection with their surgical use.

The fibrin clot in mammalian blood plasma forms when, as a result of the catalytic action of thrombin, the soluble protein fibrinogen changes to the insoluble protein fibrin which then precipitates out of solution.<sup>7</sup> Plasma clots have been found, by microscopic examination, to be composed of interlocking needles,<sup>7</sup> and by x-ray spectrography, each needle has been shown to be built up in a definite crystal structure out of regularly oriented molecules.<sup>16, 17</sup> Thus far, however, no information is available from which it is possible to determine definitely whether the breaking of a clot under tension is the result of the rupture of needles in the fibrin mesh or of the separation of the individual fibers. In the one case the orientation of individual molecules in a fiber, in the other the orientation of fibers in the mesh, would be the important factor in determining the tensile strength of a clot. It is possible that both factors are involved.

Attempts to eliminate the factor of variability when determining tensile strength by modifying the method of preparing and testing the clots were unsuccessful. For this reason the variability may have to be considered as an intrinsic property of the clots. It is a common observation in testing the elastic and mechanical properties of many substances that their stress-strain curves rise smoothly up to the neighborhood of the breaking point where they change their direction sharply. If such a phenomenon occurs in our clots, the measurement of their tensile strength is complicated by changes occurring during the breaking process. Accurate stress-strain curves are necessary to determine how important this type of phenomenon is in causing the variability of our data. On the other hand, the variability in the tensile strengths of plasma clots may also be affected by the complexity of the mechanism of fibrin formation and the conditions of its precipitation.

The rate of thrombin formation may conceivably play an important role. Quick<sup>7</sup> has pointed out that the amount of thrombin required to coagulate plasma in five minutes is only slightly greater than that required to produce the same effect in several hours. Nygaard<sup>10</sup> by means of photoelectric studies on the coagulation of recalcified citrated plasma has shown that the duration of the period of fibrin formation increases when the time for the onset of coagulation is prolonged. This means that relatively minute differences in the rate of conversion of prothrombin to thrombin such as might be caused by minor differences in the amount of incidental injury to platelets in pipetting and manipulation may cause marked differences in the time required for the conversion of

fibrinogen to fibrin, perhaps with corresponding differences in the structure and arrangement of the fibrin strands, and consequently in the tensile strength of the clot.

It is known that the pattern of crystallization of cupric chloride as a result of the drying of solutions mixed with hemolyzed blood varies remarkably with the blood of different individuals in different pathologic states.<sup>18, 19</sup> It would, therefore, not be surprising should the pattern of crystallization of the very much more complex fibrin molecule be found to be even more sensitive to as yet undetermined factors in the blood plasma.

#### SUMMARY

1. The mean tensile strength of plasma clots is proportional to the cross-sectional area of the tubes in which they are formed.

2. The nature of the surface of the test tube in which clots are formed influences the tensile strength of the plasma clots to a slight extent.

3. The mean relative tensile strength of plasma clots formed at 10° C. and 20° C. is lower than that of clots formed at 37° C.

4. Plasma clots incubated aseptically at 37° C. with precautions against evaporation of fluids and dissolved gases gradually weakened during a testing period of eight days.

5. By a comparison of the tensile strengths of groups of clots prepared from anticoagulant-containing plasmas by adding appropriate amounts of coagulants with the strengths of unmodified plasma clots prepared from the same blood samples, it has been demonstrated that:

A. Clots prepared from unmodified plasma diluted with saline solution are weakened approximately in proportion to the amount of dilution.

B. Clots prepared from unmodified plasma to which muscle and lung extract are added coagulate more rapidly than the controls, but are weakened by more than the dilution factor. Lung extract reduces the tensile strength more than muscle extract.

C. Clots prepared by recalcifying plasma obtained from citrated blood are weakened to an extent roughly proportional to the dilution of the plasma. Clots prepared by adding suitable amounts of the citrate and calcium chloride in dry form to unmodified plasma are as strong as those prepared from unmodified plasma.

D. Clots prepared by recalcifying plasma obtained from oxalated blood are weakened to a slightly greater degree than would be expected on the basis of dilution alone. Plasma to which oxalate and calcium chloride were added in dry form also yields clots which are weaker than their controls.

E. Clots prepared by adding rabbit "Globulin" to the plasma obtained from heparinized blood are weaker than would be expected as a result of dilution.

F. Clots prepared by adding protamine sulfate solution to the plasma obtained from heparinized blood show no weakening as compared to the controls:

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# LABORATORY METHODS

## GENERAL

### APPARATUS FOR CONSTANT RATE INTRAVENOUS INJECTION OF ULTRA SHORT-ACTING ANESTHETICS IN MICE\*

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THE apparatus to be described, consisting of a mouse holder and injection machine, was designed to facilitate constant rate intravenous injections in mice of ultra short-acting anesthetics. The mouse holder (Fig. 1) is modified from Burdon's<sup>1</sup> design and consists of a wire and metal cage, hinged at the forward end (1) and held down at the rear by a lug (2) on the right side. The front is closed by a plate which adjusts lengthwise for different sized mice, and the back is made of another plate soldered to the cage and providing a hole for the tail. The mouse holder is mounted on a paraffined wood block, which is supported by a 7 inch length of  $\frac{3}{4}$  inch threaded iron pipe. Screwed over the pipe are two flanges, one facing upward and attached to the wood block by screws, and the other (3) facing downward and resting on the table top. The height of the holder is adjustable by screwing the bottom flange up or down, while rigidity is insured by a washer and nut on the pipe projecting beneath the table. Ordinarily a height is used which will just allow a 150 c.c. beaker beneath the tail of the mouse. It is our experience that immersing the tail for about one-half minute in water at 45° C. gives the best dilatation of tail veins.

The injection machines described in the literature<sup>2-5</sup> are not very satisfactory for the ultra short-acting anesthetics because of the presence of one or more objectionable features; namely, pulsating flow, uncertain control of rate, lack of provision for continuous variation between different rates over a wide speed range, and the difficulty of adaptation of the machine so that large numbers of mice can be easily and quickly injected with small accurately measured volumes.

The present machine (Fig. 1) consists of a source of power, variable speed device, flexible drive, screw, and syringe clamp. An inexpensive rim-drive phonograph turntable, which comes complete with synchronous motor, supplies the power. The turntable (19), from which the felt was removed by soaking in hot water, supports a steel wheel (18), on the rounded rim of which is cemented a wide rubber band (16). The axle (15) of this wheel rests in open slot brass bearings (14, 17) on each side of the turntable, and these slots are adjustable vertically to permit the use of different sized wheels. A  $\frac{3}{4}$  inch length of speedometer cable (12) is sweated into two sleeves, one of which (13) receives

\*From the Department of Pharmacology of the Vanderbilt University School of Medicine.  
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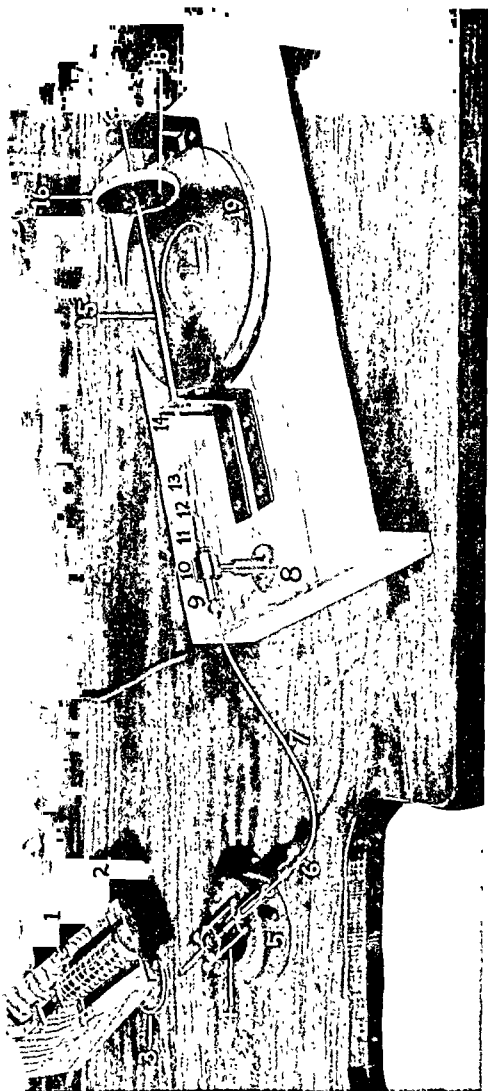


FIG. 1—NOTE: To simplify construction the handmade ball bearing shown above (10) can be replaced by a standard bearing described in the text and illustrated in Fig. 2.

the axle, while the other (11) slips over a short steel rod which is snugly fitted inside a double row ball bearing (10). This bearing (Fafnir No. 5200), Fig. 2, is held by a setscrew inside a piece of steel pipe, which is counterbored to let the bearing in against a shoulder and tapped beneath for a supporting rod adjustable for height.

In the syringe clamp and screw assembly (Fig. 3), the plunger of a  $\frac{1}{4}$  c.c. syringe is driven by a screw connected to the rod within the ball bearing by a 1 foot length of fairly stiff flexible cable (7 in Fig. 1), held at each end in chucks (6, 9 in Fig. 1). Cable and chucks are Cenco No. 18812. The screw, 32 threads per inch, runs through a thread and is steadied by a small brass bearing. The syringe is held in a brass clamp which is fastened to the screw mechanism by two knurled nuts. A small spring bears lightly against the shaft of the plunger and helps to hold the latter fixed after adjustment of the dose has been made. Removal of the knurled nuts permits the syringe with its clamp to be detached for cleaning without disturbing the alignment, and with additional clamps, rapid interchange of several syringes containing different solutions is facilitated.

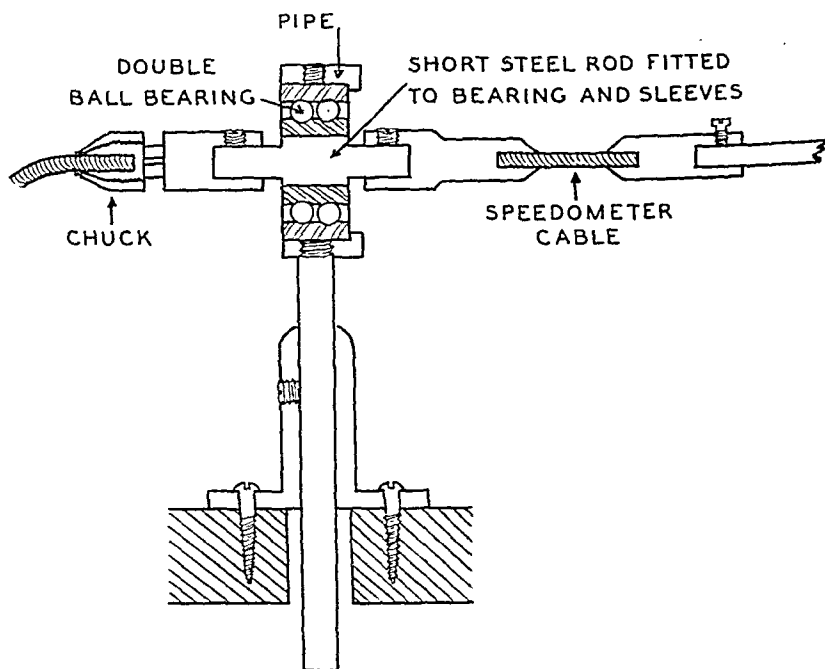


Fig. 2.—Cross section view of ball bearing, support, and drive shaft connections.

The position of the injection machine platform relative to the mouse holder is adjusted so that the flexible cable (7) lies in a quarter circle. This allows the right hand to lie under the cable and grasp the syringe from the right, while enough slack is left in the cable so that as the screw revolves it can take the cable forward.

The dose adjustment is made by lifting the axle (15) to free the wheel (18) from contact with the turntable, running the screw back by hand, and drawing the solution into the syringe past the desired mark. Then the screw is similarly

run forward until the plunger reaches the exact mark, which can be attained within 1 or 2 thousands of a cubic centimeter. During this latter procedure, in order to avoid dosage errors, the syringe clamp and screw assembly (4) is held flat. In that position, it is then laid down on a cork ring (5), and the injection is made later without allowing the clamp to rotate to the left, since this would cause a premature ejection from the syringe. In practice, this precaution has proved easy to observe.

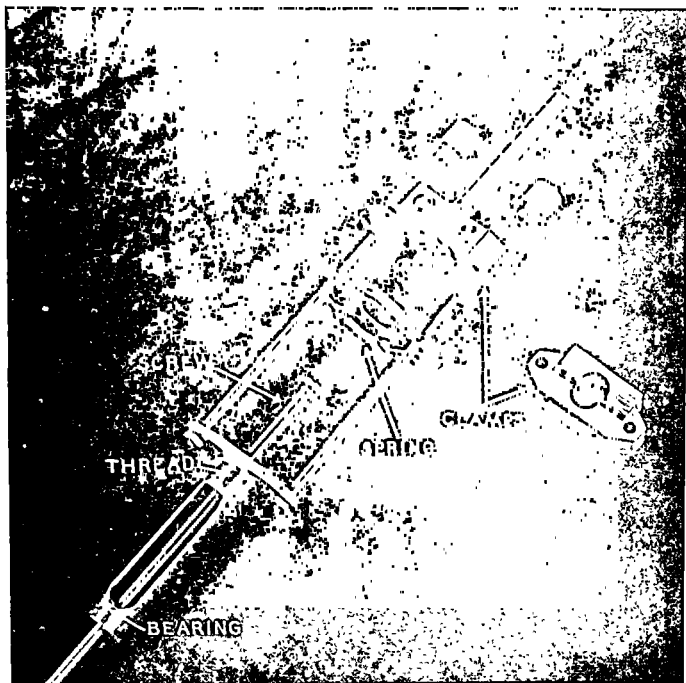


Fig. 3—Syringe clamp and screw assembly

When the electric current is turned on by means of a footswitch, the speed of the turntable reaches its maximum in about a second. If this lag should be found objectionable, it could be practically eliminated by mounting a solenoid under the right end of the axle (15), and with the turntable running continuously, by starting and stopping the injection through the vertical movement of the solenoid core acting to lower or to raise the wheel (18). The speed of the injection can be varied over a range of from about 0.002 to 0.04 c.c. per second, by using two interchangeable wheels (18),  $1\frac{1}{2}$  inch and 3 inches in diameter, and

varying the wheel position on the turntable. A steel mechanic's rule graduated in 0.5 mm. divisions is quite satisfactory for determining the wheel position. The exact error in rate for rapid injections, lasting only a few seconds, is difficult to determine with a stop watch; but with longer injections, for example, thirty seconds, the error is less than 2 per cent.

At the end of injection, as the plunger seats home, the clamp begins to rotate clockwise in the hand, which insures a full dose and also acts as a signal to step on the footswitch and shut off the motor. There is very little danger of breaking the syringe or plunger, since the flexible components of the drive take up the shock, which if prolonged, merely causes the wheel to skid on the turntable. The only occasion on which a syringe plunger broke was when the syringe was not well aligned with the screw. This alignment can be improved if necessary by shimming with small pieces of paper at the joint where the syringe clamp is attached to the screw mechanism by the knurled nuts. It may be found necessary to grind off the head of some plungers or cement a small piece of cardboard on the head to true it up in relation to the adjoining face of the screw.

This machine has been used in about 2,000 intravenous injections in mice, and presents the following advantages: (a) The dose can be quickly and accurately measured in a power-driven syringe which retains the necessary maneuverability for direct injection of tail veins. (b) The injection proceeds at a smooth and reproducible rate which can be varied continuously over a considerable range. (c) The machine is relatively simple and inexpensive to construct.

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## A NEW HEMATOCRIT TUBE

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THE author has recently reviewed a publication on the anemia of flexed-tailed mice by Grüneberg (1942) in which a new hematocrit tube was described. This tube has proved so satisfactory, and in some respects superior to the hematocrit tubes in common use, that it is thought wise to draw it to the attention of workers in this country.

A diagram of this hematocrit tube is given in Fig. 1. The merits of this tube are that it requires such a small quantity of blood and that it has an enclosed end, and thus leakage during centrifugation, which so often occurs in the many modifications of the open end tubes, is completely prevented. The Wintrobe hematocrit tube also has a sealed end, but requires more blood than is often available from small animals or even large animals unless venepuncture is used. The size of the tube is such that it fits into a standard 50 c.c. centrifuge cup with a reducing cap. The capillary bore stem may be made in a variety of sizes depending on the amount of blood available. The author has two sizes for use in determining the packed cell volume of small laboratory animals. In one, the calibrated stem contains 20 c. mm., and the other 0.1 c.c. to be used with 20 c. mm. and 0.1 c.c. of blood respectively. The stem is divided into 50 equal parts, thus permitting the determination of the packed cell volume within 0.5 per cent by estimating the nearest quarter of a division. The volume of the packed cells read directly from the stem of the tube multiplied by two gives the volume of red cells in percentage. If desirable, the stem may be calibrated from 0 to 10 instead of 0 to 5 as here indicated, in which case the percentage of packed cell volume may be read directly.

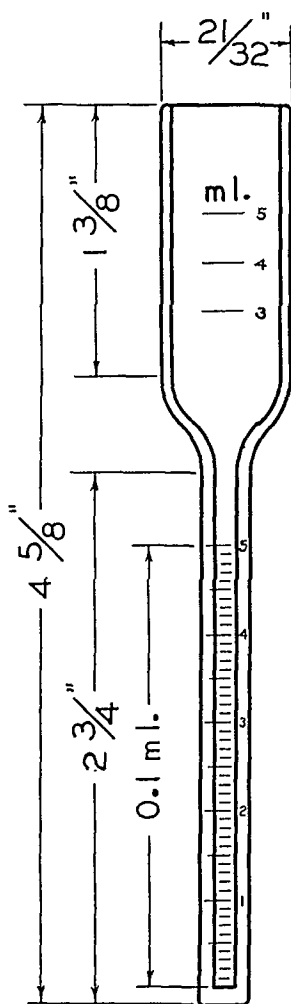
The method of the author has been as follows: Deliver about 3 c.c. of isotonic saline into the hematocrit tube by means of a syringe with a 5 inch, 18 gauge needle which permits filling the tube from the bottom of the stem. If the hematocrit tube is a 0.1 c.c. one, deliver 0.1 c.c. of blood into the saline from a pipette calibrated to contain 0.1 c.c.; rinse the pipette two or three times with the saline and centrifuge to constant volume. Usually centrifuging at 2000 r.p.m. for one hour is sufficient. The tube may be conveniently cleaned by flushing out the stem and cup by means of the syringe and long needle.

For some time 0.85 per cent saline has been considered isotonic to mammalian blood, but in light of the recent work of Aldred (1940), this figure needs revision upwards. Aldred by use of the vapor pressure technique has determined the osmotic pressure of the blood of various animals and found that in the majority of mammals 0.93 per cent saline is isotonic to blood. Grüneberg found 0.93 per cent saline isotonic to mouse blood. Which one of the saline solutions selected is probably not as important as is a uniform use of one or the

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other, inasmuch as the centrifuge hematocrit is only a close approximation of the true red cell volume, and thus its use is for comparative purposes (Chapin and Ross, 1942).



## HEMATOCRIT TUBE

Fig. 1.

Several sets of hematocrit tubes have been made for the author by Will Corporation, Rochester, N. Y., but any supply house should be able to furnish them to order. For precise work, the tubes may be checked for accuracy by calibrating the stem with mercury.

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## RABBIT BOX\*

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IN ORDER to facilitate the handling of large groups of rabbits in testing samples of human serum albumin for pyrogenic activity, a rabbit box has been

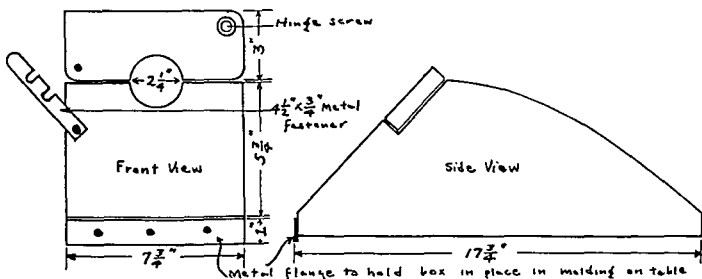


Fig. 1.

Fig. 2.

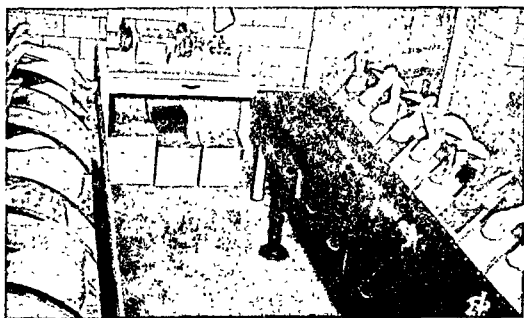


Fig. 3.

designed which offers some worth-while advantages. The animal remains in the box throughout the period of observation without being disturbed and without any noticeable discomfort. Incidentally, this type of box is also quite useful for skin-testing experiments, injection work, and blood-sampling experiments.

\*From The Lilly Research Laboratories, Eli Lilly and Company.

The original idea for a box of this type was obtained while visiting the Massachusetts State Antitoxin and Vaccine Laboratory several months ago.

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Fig. 1 shows a detailed drawing of the front of such a box, while Fig. 2 shows a side view. Seven-eighths inch wood is used for construction, and the rounded edges as shown are made to allow the boxes to be placed against one another in a row. The entire top of the box is open as can be seen in Fig. 3.

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## A SIMPLE RAPID METHOD FOR PARAFFIN TISSUE PREPARATIONS UTILIZING THE KAHN SHAKER\*

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LT. COLONEL FERDINAND C. HELWIG, M. C., A. U. S.

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THE almost universal desire for rapid tissue diagnosis has resulted in numerous modifications of well-known methods of fixation, dehydration, and paraffin infiltration. In the hands of many workers these modifications, designed to decrease the time element, have frequently resulted in incomplete dehydration and infiltration, making cutting difficult. Moreover, the sections, thus obtained, all too often have shown too much shrinkage, poor staining qualities, and other objectionable features.

For some time now we have been employing a method in this laboratory which is not only rapid, but at the same time permits us to make histologic preparations that are easily sectioned and when stained show remarkably little shrinkage, less in fact than we have observed by the slow methods. Furthermore, we have eliminated certain common, more or less standard, steps which have likewise accelerated the preparation of very satisfactory sections.

It is possible by this method to make excellent sections, ready for examination within five to six hours after the tissue is received in the laboratory in the fresh state.

### DESCRIPTION OF METHOD

Blocks not over one and one-half millimeters thick or one and one-half centimeters in diameter are cut from the tissue to be processed. If the tissue is fresh and unfixed, it can be suitably hardened by shaking it in the Kahn shaker in 20 per cent solution of formaldehyde for twenty or thirty minutes. We have been using small twenty cubic centimeter black screw-topped glass vials eight centimeters long and two centimeters in diameter. These vials are placed horizontally in the Kahn shaker so that the tissue will undergo constant agitation and will also be subjected to the largest, constantly changing, volume of fixing or dehydrating agent possible. If great haste is essential some time may be saved by immediate fixation in hot 4 per cent formaldehyde, but this method is undesirable since undue shrinkage and distortion result. Routine material can be fixed in the usual manner in 4 per cent formaldehyde solution for eighteen to twenty-four hours.

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\*From the Laboratory Service Army Air Forces Regional Station Hospital 1, Coral Gables, Fla.

After fixation the tissue blocks are blotted and the following solutions are employed:

SOLUTION	TIME
95 per cent Ethyl alcohol	30 minutes in Kahn shaker
Absolute alcohol	15 minutes in Kahn shaker
Acetone	15 minutes in Kahn shaker
Chloroform	30 minutes in Kahn shaker
Chloroform and soft paraffin (equal parts)	60 minutes in 57° C. incubator
Hard paraffin	60 minutes in 57° C. incubator

Absolute alcohol should be employed no more than three times and may then be used in place of 95 per cent alcohol for the first dehydration. Acetone should be used no more than three times. It may then be dehydrated over anhydrous copper sulfate and used over and over again.

The containers used for the fixing and dehydrating solution should contain at least fifteen cubic centimeters of the reagent for each tissue block. Tissues are embedded in hard paraffin nine parts and bayberry wax one part. A long block is made using a double "L" type lead embedding box. No fiber blocks are necessary since the paraffin bayberry wax mixture, when hardened into a block, can be screwed into the microtome and the blocks section easily. Routine sections are cut in our laboratory at six microns. Sections are then affixed to glass slides in the usual manner and stained by any of the methods suitable for paraffin preparations.

*Comment.*—The procedure just described is suitable for both surgical and necropsy material; it is saving of reagents and is rapid. In our hands this method has yielded uniformly good results over a long enough period of time and with a sufficiently wide variety of tissues to convince us that it is suitable for more widespread application.

## A MEDIUM FOR DETERMINATION OF CITRATE UTILIZATION BY COLON BACTERIA\*

CAPT. W. BLAKE CHRISTENSEN,† U. S. ARMY

DURING an investigation of certain unclassified dysentery bacilli in Tunisia, North Africa, it became desirable to test the reactions of the organisms on media containing citrate as a sole source of carbon. Since neither Simmons' citrate nor Koser's citrate nor the materials to make them up were available, it was necessary to devise a modification. A medium of the following composition was found suitable:

Ammonium sulfate (Merck Reagent)	1.0 Gm.
Magnesium sulfate (Merck U.S.P.)	0.2
Monopotassium or dipotassium phosphate (Merck Reagent)	1.0
Sodium citrate (Merck U.S.P.)	3.0
Sodium chloride (Merck Reagent)	5.0
Agar	20.0
Phenol red	0.012
Distilled water	1000 c.c.

The medium is heated to dissolve the agar, and the reaction is adjusted to pH 7. If monopotassium phosphate is used, alkali must be added, but if the dipotassium salt is used, acid must be added to bring the pH to 7. The medium is tubed and sterilized at 15 pounds pressure for twenty minutes and allowed to solidify in the form of slants. The fresh medium has a pale, yellow-orange color. If a liquid medium is desired, the agar may be omitted.

Organisms which utilize citrate as a sole source of carbon produce, within twenty-four hours on the solid medium, a yellow color in the butt and a very striking, intense violet color on the slant. Organisms which fail to utilize citrate leave the medium unchanged. In the liquid medium, citrate-positive organisms produce acid within twenty-four hours, with perhaps a narrow alkaline zone at the top of the tube. Within forty-eight hours, however, the entire body of the medium changes to the characteristic intense violet color. The presence of the indicator in the liquid medium eliminates the necessity of estimating growth by turbidity, as was done in the original Koser medium, and as large an inoculum as desired may be used.

Both liquid and solid media have been tested with 162 different strains of nonpathogenic and pathogenic colon organisms. The results are shown in Table I. It is seen that only organisms of the *Aerobacter* and colon intermediate groups, and *Salmonella typhi murium* utilized the citrate. Organisms of the *Escherichia*, paracolon *Escherichia*, and *Shigella* groups, and *Eberthella typhosa* failed to utilize the citrate. In the anaerogenic paracolon group only citrate-negative organisms were found, no doubt because of the small number of strains tested.

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†Chief of the Bacteriology Section, Sanitary Corps.

Though it would have been desirable to check organisms of the colon intermediate and the anaerogenic paracolon groups with standard citrate media to determine the possibility of false reactions, it was manifestly impossible to do so. However, the uniform negative results obtained with organisms known to be citrate-negative and the positive results with organisms known to be citrate-positive are ample evidence of the reliability of the medium.

TABLE I

CITRATE UTILIZATION BY COLON ORGANISMS ON THE MODIFIED CITRATE MEDIUM

ORGANISM	NO. OF STRAINS TESTED	CITRATE UTILIZATION	INDOLE	VOGES-PROSKAUER
<i>Escherichia</i>	46	-	+	-
Paracolon <i>Escherichia</i>	5	-	+	-
Colon	8	+	+	-
Intermediate	4	+	-	-
<i>Aerobacter</i>	24	+	-	+
Anaerogenic paracolon	2	-	+	-
	3	-	-	-
Various <i>Shigella</i>	23	-	+	-
Species	45	-	-	-
<i>Salmonella typhi</i> <i>murium</i>	1	+	-	-
<i>Eberthella typhosa</i>	1	-	-	-

- Negative.

+ Positive.

Considerable stress has been placed on the utilization of chemicals of the highest possible purity in a citrate medium. For this reason, some objection may be made to the use of the two U.S.P. salts in the medium. However, even if the impurities consisted entirely of a compound utilizable by citrate-negative organisms, and if the utilization brought about the same type of reaction as true citrate fermentation (assumptions which are in themselves very unlikely), there still would not be enough of the contaminating compound present to produce enough alkali to change the color of the medium. Impurities would hardly be present in a concentration to exceed 0.0005 M. (based on stated impurities in the Merck Index, 1941). Such a concentration is well below the known affinity constants of enzymes, and the compound would not be used at all, or would be used so slowly that appreciable growth would not take place.

The medium described consists of materials that are cheap and readily available in most laboratories. While it will probably not replace standard media on the market, it will fill the needs of laboratories which use such a medium rarely and thus have no reason to lay in a stock of the prepared product.

## A MULTIPLE RABBIT HOLDER\*

EDWIN P. LAUG, PH.D., WASHINGTON, D. C.

**I**N STUDIES requiring the immobilization of rabbits for extended periods, it has often been found that the usual methods are undesirable, because they may produce either (1) stasis from the tie strings (even when padded); or (2) broken legs and backs due to struggling.

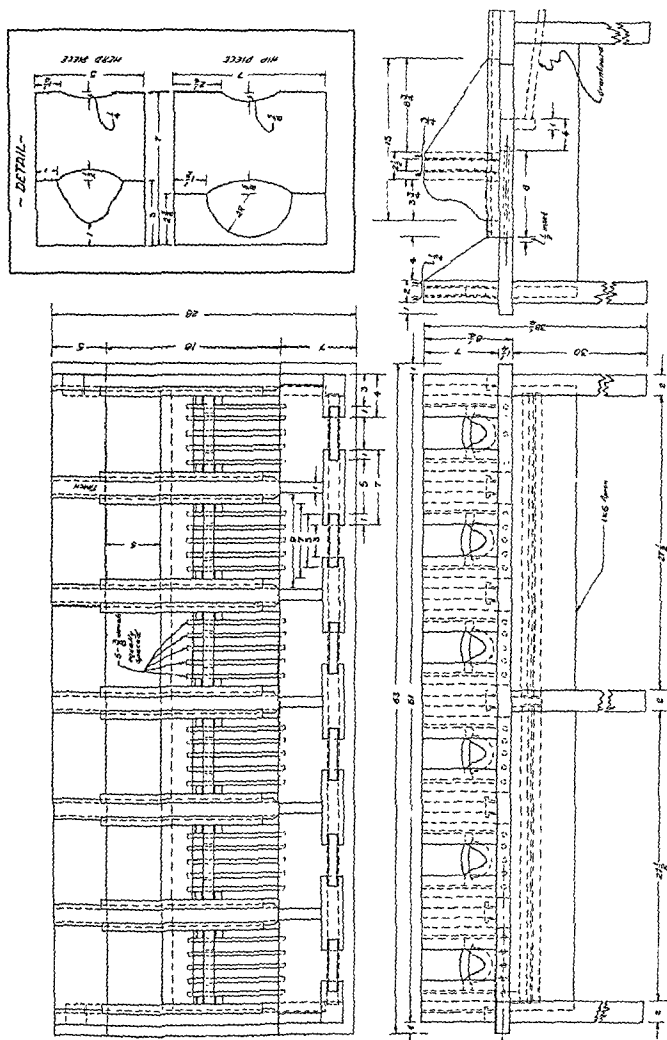
These difficulties have been overcome through the design of a holder which immobilizes the animal after the manner of a head and hip stock, but without restriction to the movement of fore or hind limbs. The animal rests comfortably on its belly; but because of the fact that its hind quarters are held rigidly, no leeway is permitted for sufficient arching of the back or other motion to cause injuries which so frequently result. The type of holder described here has been in use for nearly two years; during this time approximately 2,500 rabbits have been kept in the holders for periods varying from one to twenty-four hours. In this extensive experience, practically no injuries have resulted when the animals were properly placed in the stocks; occasionally accidents have occurred where the confining jaws were improperly adjusted so that the animal managed to free either the head or the hips. Constructional details: In Fig. 1 are reproduced the constructional details for a 6-rabbit holder. For long service the use of a hardwood such as birch or maple is recommended, particularly in those parts subject to frictional wear. It is not desirable to describe the construction in any detail, since it is believed that the pictures and drawing are self-explanatory. Several features, however, may well be stressed:

The head piece is stationary, being part of the framework of the holder. Both jaws of the head piece are removable, and this facilitates cleaning. In practice it has been found most essential that the lowest point in the opening of the lower jaw piece be fixed not higher than one inch above the table level upon which the animal rests; otherwise the head is elevated too much and choking results. Adjustments for size of the head opening are made by locking the upper jaw piece in proper position through a series of nail holes, one of which passes through the stock frame. The hip stock is adjustable for the size of the animal's hips in a manner similar to the head stock. In addition, however, the entire hip stock is movable forward and backward, sliding in two groove tracks, one on each side. Thus adjustments for the length of the animal can be made quickly. As in the case of the lower jaw piece of the head stock, it is very essential that the lowest point of the lower hip jaw be not further than  $\frac{1}{2}$  to  $\frac{3}{4}$  inches above table level. Failure to observe this dimension leads to serious constriction, and bladder injury often results. The openings of the head and hip jaws have been adjusted to accommodate animals weighing 2 kilograms or over.

\*From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency.

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NOTE. ALL DIMENSIONS IN INCHES  
FIG. 1.

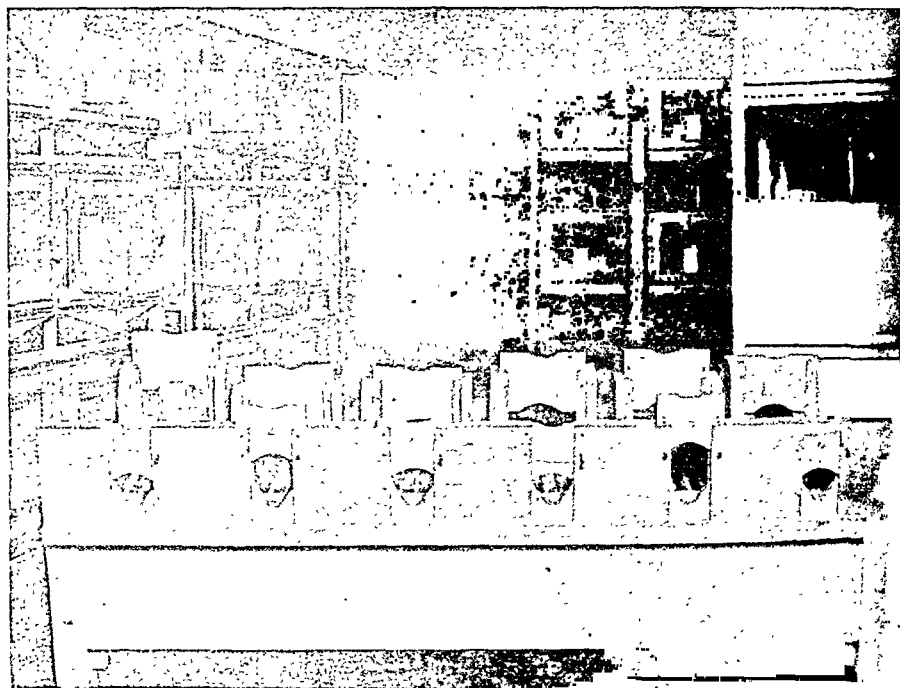


Fig. 2.



Fig. 3.

The shape of the fixed lower jaws of both head and hip stocks will accommodate animals weighing over 5 kilograms. For small animals, the top jaws of both head and hip stocks have been made reversible (see Fig. 1). For very small rabbits (less than 2 kilograms) further modification of the shape of the top jaws would be necessary.

The belly of the rabbit rests on a framework of 5 dowel pins. This feature is extremely useful where fluids are applied to the skin of the back. Sometimes these tend to run off and without the open construction would collect under the belly of the animal.

At the back of the holder, provision for removal of excreta has been made by installing a removable drain board as shown. Quantitative collection of urine and feces can, of course, also be made by substituting for the drain board a sliding rack for glass baking dishes and a baffle for deflecting urine.

The length of time that an animal can be kept immobilized becomes primarily a problem of a need for food and water. Usually rabbits will not eat voluntarily when thus confined. While held in the holder, however, it is relatively easy to pass a stomach tube.

While the author has had no experience with other animals, it would seem feasible to modify the sizes of the head and hip jaws to accommodate dogs and cats.

## A SOLID MEDIUM FOR THE TRANSPORTATION OF DELAYED GONOCOCCUS CULTURES\*

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THE provision of reasonably adequate facilities for gonococcus cultures in a state laboratory requires the solution of several problems: First of all, the establishment of reliable local laboratory service and, second, the development of a method for sending gonococcus cultures through the mail. The former depends on a simple method for teaching technicians, many of whom are not familiar with bacteriologic procedures, to do such work. The latter depends on the establishment of a method for keeping the gonococci alive and holding back contaminating organisms. Many such methods have been described, using special liquid media, ice or dry ice refrigeration, or "thermos" bottle containers.<sup>3, 4, 5, 8, 9, 11, 12, 13, 19, 21, 24, 26</sup>

It is well known that diagnosis of gonorrhea in the male can be made by examination of suitable smears. In the female, however, especially in chronic or treated cases, a few doses of sulfonamide may render the smears negative, while many viable organisms are present, or the large number of contaminating bacteria may interfere with the interpretation of a smear, even if care is taken to take these smears from the cervical canal.

The value of gonococcus culture is well known. Thomas<sup>22-24</sup> has summarized this work through 1941. Even with the relatively ineffective means now available, cultures yield two to three times as many positive diagnoses as smears in chronic or treated female cases.<sup>14, 15, 16, 17, 20, 25</sup>

North Carolina is a rural state, approximately 550 miles wide, with considerable variations in temperature, ranging from zero in the mountains in winter to 103° F. during the summer. While 87 of its 100 counties have organized full-time health departments, most of the culture material will come from well-trained physicians in urban centers, country doctors and nurses in rural centers, and will take from two to three days to reach the laboratory.

For this reason, it seemed inadvisable to use liquid medium or to refrigerate specimens with ice or dry ice. We have, therefore, attempted to develop a suitable solid medium in tubes to be inoculated with material collected on full size applicator swabs. This paper is a preliminary report of the results obtained using such a medium.

Preliminary work was done on 25 specimens, secured from untreated prostitutes from the Woman's Prison in Raleigh and from nearby clinics sufficiently close to Raleigh that the specimens might be brought by messenger. Later, 117 specimens were secured from 85 patients in a well-directed Gonorrheal Clinic, conducted by Dr. Margery J. Lord, City Health Officer, Asheville, N. C., some 270 miles from Raleigh. It was felt that cultures which could survive

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this long trek across the country in the mails should be a fair test of a method suitable for the entire state.

The mailing media consisted of numerous combinations of blood with and without the addition of dyes and other substances to inhibit contaminants.<sup>3, 4, 5, 6, 10, 21</sup> Each one was tested for mailing qualities, inhibition of contamination, and preservation of the gonococcus. Those combinations containing less than 1 per cent agar did not stand transportation and were eliminated. In the development of the media various concentrations of gelatin and agar were tried, the former ranging from 2 per cent to 24 per cent, the latter from 0.1 per cent to 2.5 per cent. Agar by itself is too brittle; when a swab is introduced into it, even if the medium contains up to 50 per cent blood, the agar is broken up with large air spaces resulting. It was found that the addition of 4 per cent gelatin to 2 per cent agar produced a medium of smooth composition, regardless of the quantity of blood, into which a swab can be introduced and withdrawn with ease.

Each mailing medium was tested with a known strain or several known strains of the gonococcus. The media containing inhibiting agents were tested with known cultures of the colon bacillus, *Staphylococcus aureus*, and proteus. Concentrations of dye which do not kill the gonococcus are for the most part ineffective against the latter organisms. Almost all the combinations showed some inhibition of contaminants but did not exhibit the marked inhibition which is probably desirable. A few tests were made with tyrothricin<sup>2, 21</sup> which seemed no better or no worse than Nile blue A.<sup>10</sup> Crystal violet of suitable dye content as suggested by Cox<sup>4, 6</sup> was tested also, and a few tests using brilliant green, alone or in combination with the other dyes, were tried. (Dr. Cox very kindly sent us some of his media to try.) It seems that the degree of contamination in the final cultures depends more upon the bacterial flora of the patient than upon the inhibiting agent used. The isolation of the gonococcus was made both from heavily contaminated and slightly contaminated plates. We do not know how much the contaminants interfere with the preservation of the gonococcus. It does not seem that sulfonamide treatment of the patient interfered to any large degree with the isolation of the organisms.<sup>19</sup> Para-amino benzoic acid was introduced into one set of mailing media, but better results were obtained without this addition. The medium which we have finally determined as the most suitable to date consists of the following: proteose-agar No. 3 (Difco), 4.5 per cent plus additional shredded plain agar to make a final concentration of 2 per cent, 4 per cent gelatin, 0.24 per cent Nile blue A, and 20 per cent chocolate blood. The medium is very smooth, easy to penetrate with a swab, and supports the growth of the gonococcus luxuriantly. It seems to be more satisfactory with Nile blue A than with gentian violet, tyrothricin or brilliant green, although it must be stressed again that all evidence in our series of cultures point to the fact that no satisfactory bacteriostatic agent has yet been tested, the degree of contamination depending for the most part upon the patient's vaginal flora.

Cultures and smears were taken as the patients were examined in the routine Friday night Asheville Gonorrheal Clinic. (We are grateful to Dr. Lord for this clinical material.) Some of the patients were new acute cases, some were under treatment, others were recurring cases which had received

treatment sometime before, and a few were new contacts. Swabs were taken from the urethra, using pressure on the Skene's Glands to milk out any material present. Smears and cultures were made from these swabs. A speculum was then inserted in the vagina, the cervix cleaned off with a cotton swab, the mucous plug in the cervix expressed, and another smear and culture were made with a swab from the cervical canal. The clinical condition of the patient was carefully noted.<sup>15</sup> This procedure is followed routinely in the clinic and gives approximately three times as many positive cultures as smears.<sup>16</sup> To test the mailing media, additional swabs were taken from the cervix and in some instances also from the urethra, using sterile forceps to break off the swabs and push them down into the mailing tubes.

After the clinic, that is about ten o'clock at night, these mailing tubes were labeled and packaged to be sent through the mail the next morning to the State Laboratory of Hygiene in Raleigh. Occasionally the package was received at the laboratory on Sunday, in which case cultures were made immediately. Generally, however, the tubes were received following the sorting of the mail on Monday and were cultured Monday afternoon, approximately three days after the swabs had been taken from the patients. In one series five days elapsed. In the group of cultures tested before this routine was established in the Asheville Clinic, specimens remained in the mailing media from twenty-four hours to six days. The mail between two points probably never takes that long, but cultures were purposely held in the laboratory to see how long they would remain positive.

The swabs were removed with sterile forceps, streaked onto Peizer's medium<sup>18</sup> and onto chocolate agar<sup>2</sup> plates. These plates were sealed in a jar containing a moist piece of cotton and a lighted candle and incubated for two days at 36° C. Some of the swabs were suspended in 1/2 c.c. of 2 per cent proteose peptone No. 3 solution, well-shaken, and cultures were made from this suspension. It was noted that growth on the surface of some of the mailing media looked suspicious for the gonococcus, and so these tubes were incubated in the jars with the subcultures after the swabs had been removed and were tested by the oxidase reaction two days later. A few positives were obtained by this method.

After forty-eight hours, the plates were examined by means of the oxidase reaction and Gram-stained smears. Doubtful strains were tested by fermentation reactions. The plates were read carefully for the degree of contamination as well as for the number of positive colonies which in most cases was low, occasionally one or two oxidase positive colonies only appearing on the surface of the plates. The results obtained in this laboratory were checked against those control cultures and smears which had been made in the Asheville City Health Department Laboratory at the same time.

Table I shows the results from our series of 142 cases. Of the 25 specimens received by messenger from patients presumably with gonorrhea, 10 showed immediate positive control cultures, while 8 delayed cultures were positive, 3 after one, 3 after two, 1 after three, and 1 after four days. Most of them remained on the desk in the laboratory for the specified period of time, while a few were mailed in from near-by towns.

Forty-four specimens from cases of acute gonorrhea diagnosed by positive smear and culture were mailed to the laboratory. The immediate control cultures of the 44 cases were positive, and 31 of the delayed cultures were positive, 2 after two, 20 after three, 6 after four, and 3 after five days in the mail.

Sixty-nine mailed specimens were received from cases of chronic or treated gonorrhea. None of these showed positive immediate control cultures, 12 of them showed positive cultures from the specimens which were mailed to the laboratory, 2 after two and 10 after three days. These 12 positives possibly may be accounted for by the fact that the mailing medium may absorb some of the drugs used in treatment which may be affecting the direct growth of the gonococcus, or by the fact that in these cases there are present only a few gonococci which may be picked up on one swab and not on another or may even have been missed on the direct cultures. Four cultures from clinically negative contacts were negative throughout. These results indicate that the mailing medium is quite satisfactory for isolating the gonococcus after several days have elapsed. Since the greatest number of cultures reached the laboratory in three days, this accounts for the seemingly large number of positives obtained in that length of time. In the series of 117 mailed cultures, a total of 16 were received in two days with 3 positive isolations, 73 were received in three days with 31 positive isolations, 13 were received in four days with 7 positive isolations, and 15 were received in five days with 3 positive isolations.

TABLE I

NUMBER OF CASES	DIAGNOSIS	IMMEDIATE CULTURES		DELAYED CULTURES						
		NEGA- TIVE	POSITIVE	NEGA- TIVE	POSITIVE	DAY OF CULTURE				
						1	2	3	4	5
25 Messenger Specimens	Presumably gonorrhea	15	10	17	8	3	3	1	1	0
44 Mailed Specimens	Acute gonor- rhea	0	44	13	31	0	2	20	6	3
69 Mailed Specimens	Chronic or treated gonor- rhea	69	0	57	12	0	2	10	0	0
4 Mailed Specimens	Contacts-Clin- ically Nega- tive	4	0	4	0					
142 Total		88	54	91	51	3	7	31	7	3

## SUMMARY

A solid medium suitable for the transportation by mail of specimens for gonococcus culture has been described with the preliminary report on the use of such a medium. This solid medium stands transportation, is simple to use, and gives a fair percentage of isolations of the gonococcus.

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# CONVENIENT ASSAY METHOD FOR PENICILLIN AND SIMILAR SUBSTANCES\*

## USE OF LARGE PLATES AND FILTER PAPER CIRCLES

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THE agar cup method for evaluating the antibacterial potency of solutions is favored by many because of its simplicity and reliability. The procedure described here retains the advantages of this standard method.

The details of determining unitage of penicillin by the agar cup method (using Petri dishes) have been adequately described by Abraham et al. (1941), Foster (1942), and Foster and Woodruff (1943). The estimation of potency in penicillin solutions by the Oxford cup plate method, as carried out at the present time, involves the use of many Petri dishes in order to observe the extent of zones of inhibition over a suitable range of dilutions.

In an attempt to diminish the time and labor involved both in conducting the test and in the preparation of glassware, we have adopted the use of flat Pyrex baking dishes as culture dishes. Dishes of two sizes have been used, 6½ inches by 10½ inches by 2 inches and 12½ inches by 8 inches by 2 inches, the latter size proving the more convenient. These are fitted with plate glass covers cut to the proper size and fastened to the plate flanges with cellulose tape before incubation is begun.

### PROCEDURE

The smaller plates are prepared by pouring into them 100 c.c. of melted cooled agar seeded with 0.3 c.c. of an 18-hour culture of the Oxford test strain (*Staphylococcus aureus* strain II).

The larger culture dishes are made with 147 c.c. of agar and a 0.45 c.c. of the above inoculum. Satisfactory results have also been obtained using agar seeded with *Bacillus subtilis* spores according to the method of Foster (1942). Evaporation of excess moisture from the surface of the agar before use can be accomplished by inverting the plates and propping them open with a cork placed between one edge and the lid.

Tests are carried out by a wet filter paper method similar to that of Lamanna and Shapiro (1943). Sterile 1 cm. circles of an absorbent filter paper (Fisher No. 9-795) are dipped with flamed forceps into tubes containing the dilutions of penicillin to be tested, drained momentarily against the side of the tube and placed in rows at suitable intervals on the agar surface. A similar method has recently been used by Dowdy et al. whose work was cited by Foster and Woodruff (1943).

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A standard of known unitage is run on each plate as a control. We have found that the zone of inhibition produced by a 1 cm. filter paper circle impregnated with fluid containing 1 Florey unit per c.c. is somewhat less in diameter than that resulting when the Oxford cup plate method is used with the same standard, being, in our case, about 18 to 20 mm. The diameter of the filter paper is included in measuring the zone of inhibition.

A number of advantages are inherent in the use of the present method other than the ease of preparation of sterile apparatus. Testing numerous samples on the same culture surface increases the over-all uniformity of the assay method. Experience has also shown that the zones can be measured more quickly, there being more on each plate. A larger number of samples can be assayed by this method than by other methods hitherto described, there being comparatively little time consumed other than in making dilutions.

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## PERMANENT METACHROMATIC STAINING OF GASTRIC MUCUS SMEARS\*

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THE staining of different tissue components in different colors by a single dye is called metachromasia. Thus, if a dilute solution of a basic dye like toluidine blue is used to stain a tissue section, some of the components will be stained blue, whereas others, called chromotropic, will be stained purplish red. Typical examples of metachromasia are afforded by mucin, cartilage, amyloid, and the material constituting mast cell granules. The obvious advantage of metachromatic staining lies in the fact that it furnishes a degree of differentiation by means of one dye alone; the outstanding difficulty encountered with this technique is its lack of permanence following dehydration, a difficulty which is recognized in all textbooks of histologic technique. In our experience with the usual technique, applied to smears of mucous secretion, even air drying resulted in immediate conversion of the metachromatic purple to an undifferentiated blue. After numerous trials we finally evolved a technique which overcomes this difficulty, and we are reporting our method herewith.

The principles underlying metachromasia have been investigated mainly by Lison,<sup>4</sup> who found that sulfuric acid esters of high molecular weight are the most potent chromotropic materials. For instance, heparin appears to be responsible for the metachromatic staining of the mast cell granules (Holmgren and Wilander<sup>2</sup>); mucicetin and chondroitin sulfuric acids are the groups responsible for the chromotropic properties of mucoproteins found in mucus and cartilage. The protein moiety of the mucoproteins appears to exert no influence on the chromotropic character of these substances. The reaction is so specific that, according to Lison, it can be used as a histochemical test for the detection of the presence of these characteristic groups. Efforts to understand the nature of this color change have not been successful, however. Lison believed that there exists in the dye solution an equilibrium between the metachromatic and the normal forms, but he did not try to define the chemical basis for this differentiation; contact with a chromotropic substance can shift this equilibrium towards the metachromatic side. Other explanations have been based on differences in dye concentration between the two forms (Bank and Bungenberg de Jong<sup>1</sup>) and intramolecular rearrangements (Kelly and Miller<sup>3</sup>). None of these investigators, however, was able to explain the disappearance of metachromasia which results from treatment with alcohol or from drying.

It was our purpose to find a method for staining smears of gastric mucus so that the cellular structures would be visible by contrast against the back

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ground of mucous material. Mucicarmine alone affords no such differentiation, and when used after nuclear staining with hematoxylin, the counterstaining is erratic and generally unsatisfactory. Metachromatic staining with the usual toluidine blue technique yielded satisfactory contrasts in wet preparations, but, in keeping with general experience, the metachromatic color disappeared as soon as the smear was dried or treated in the usual way for mounting in Canada balsam; since we were no more fortunate than other investigators in formulating an hypothesis to explain this loss of metachromasia, we approached the problem in a purely empirical way and finally succeeded in evolving a technique for the preparation of permanent metachromatically stained smears of gastric mucus. Specimens thus stained ten months ago still retain their characteristic metachromatic stain.

Our procedure is as follows: Smears of fresh mucus, collected from gastric pouch dogs, are left to dry at room temperature and then fixed by flaming. The slide is then placed in the staining solution, where it is agitated vigorously for fifteen seconds and then left undisturbed for one minute more. The staining solution is prepared as follows: 1 Gm. of toluidine blue (National Aniline, certified) is dissolved in 90 c.c. of distilled water and filtered; to it are added 0.50 c.c. of HCl (specific gravity 1.18), 0.90 c.c. of  $\text{H}_2\text{SO}_4$  (specific gravity 1.84), and 10 c.c. of a 1 per cent solution of alum; finally 5 c.c. of 95 per cent alcohol are added to the entire mixture. From the staining solution the slide is taken through two changes of 1 per cent HCl (1 c.c. of HCl, specific gravity 1.18, to 9 c.c. of distilled water), being washed for about ten seconds in each change so that no more dye comes off the slide. The slide is then immersed for one minute in a 5 per cent aqueous solution of  $\text{HgCl}_2$ , washed vigorously in two changes of 95 per cent alcohol (five seconds each), and cleared in two changes of xylol. The slide should be agitated vigorously in the first change of xylol to eliminate the alcohol. Complete removal of the alcohol has occurred when the temporary blue discoloration caused by the alcohol treatment is replaced by a purple color. The slide is left for at least two minutes in the second change of xylol. Finally, the smear is mounted in Canada balsam and a cover slip is applied.

The mucus is spread on the slide gently with the aid of a glass rod, thus avoiding disruption of groups of cells and expression of intracellular mucus. The use of chemical fixatives at this stage was discarded after repeated trials with various agents. Formalin and Susa fixative both prevented metachromatic staining, saturated  $\text{HgCl}_2$  removed material from the slide and impeded the fixation of the dye after staining, acetic acid distorted the mucus by irregular shrinkage, and alcohol caused deformation of the nuclear material.

Mild acidification of the dye solution with hydrochloric acid yielded a marked increase in color contrast between the mucus and the nuclear material, but excessive acidification led to shrinkage and destruction of the nuclei. Corresponding intensification of contrast was not obtained with other acids; acetic acid, on the contrary, actually prevented the color differentiation. The addition of sulfuric acid to the dye solution, however, appeared to increase considerably the intensity and substantiality of the staining. In this connection it was observed that the use of hydrochloric acid alone resulted in an appreciable loss of dye during the later steps of the method, whereas the combination of hydro-

chloric and sulfuric acids prevented such loss. Addition of alum prevented loss of mucus from the slide and brought out sharply the intracellular mucus in a striking color contrast. The use of mildly acidulated water for washing enhanced the staining of the nuclear and cytoplasmic substances, whereas pure water imparted a purplish color to these materials, and more highly acid solutions led to a marked loss of dye.

None of the foregoing procedures, however, yielded any degree of permanence of metachromasia, unless the smear was fixed *after* staining. To this end we tried formalin (10 per cent), Susa, formol-alcohol, and saturated solution of  $\text{HgCl}_2$ ; only the last one of these gave the desired result. Its use *before* staining, however, was ineffective. The metachromasia could thus be preserved throughout the usual treatment with alcohol and xylol, and subsequent mounting in balsam even improved the brilliancy of the colors.

The finished preparations show both extra and intracellular mucus stained red to purplish red. The cells, on the other hand, are invariably stained blue, the nuclei being clearly differentiated from the cytoplasm by the greater depth of blue color. Contrast between intracellular mucus and cytoplasm is very striking. Smears prepared according to this technique permit a study of fine cellular details with the oil immersion lens.

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# CHEMICAL

## THE ASCORBIC ACID SATURATION TEST

(A PRELIMINARY REPORT)

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SINCE the isolation of vitamin C in 1928 by Szent-Györgyi,<sup>1</sup> many investigators have been interested in the chemical determination of this vitamin in blood and other body fluids. Various authors have shown that the normal adult should have a daily intake of at least 50 mg.<sup>2-5</sup> Levcowich and Batchelder<sup>3</sup> believe that the calculated ascorbic acid content of foods in a freely chosen diet may be overestimated and give a false sense of security as adequateness of vitamin C. According to Heinemann,<sup>2</sup> at least 0.8 mg. of ascorbic acid per kilogram body weight is used by a normal vitamin C-saturated individual; however, 0.4 mg. or less daily is probably sufficient to prevent scurvy. To maintain tissue saturation, 1.7 to 2.0 mg. per kilogram body weight is required.<sup>5</sup>

An individual's past intake of vitamin C may be decided either with an estimation of the ascorbic acid content of the blood or with the use of a saturation test, in which a known quantity of vitamin C is administered and the rate of excretion in the urine for a standard period of time is determined. The saturation test is based on the assumption that a past deficiency of vitamin C results in a low concentration in the tissues. Therefore, when this vitamin is administered (orally or intravenously), the tissues are capable of rapidly removing it from the circulating blood and a very small quantity is excreted in the urine. On the other hand, if the tissues are saturated, then, when the test dose is administered, the ascorbic acid will be removed from the blood and excreted in the urine in varying quantities, depending on the degree of tissue saturation. Lewis et al.<sup>6</sup> found that the renal threshold for ascorbic acid in twelve normal adults ranged from 1.1 to 1.8 mg. per 100 c.c. Faulkner and Taylor<sup>7</sup> agree that this threshold is in the vicinity of 1.40 mg. per 100 c.c. of serum.

A saturation test may be preferable to a blood determination because a venipuncture is not required. Furthermore, as stated by Harris,<sup>11</sup> "a subnormal level in the blood probably gives less clear quantitative evidence of the exact extent of the deficit. . . ."

There are objections to the use of the term "tissue saturation" as applied to ascorbic acid concentration in tissues. Hou<sup>8</sup> observed that ascorbutic tissues were capable of removing more ascorbic acid from a solution than normal tissues.

\*From the Department of Physiological Chemistry of the Naval Medical School, National Naval Medical Center, Bethesda, Md.

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NOTE: The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

The saturation test described by Harris<sup>9</sup> consists of a test dose of 700 mg. per 10 stone body weight (140 pounds), which is administered orally at 10:30 A.M. During the peak of the vitamin excretion, 2 to 4:30 P.M., a 2½-hour urine specimen is collected. This test dose is repeated daily until the body's reserves approach saturation, which is indicated by the rise of the ascorbic acid excretion. The saturated individual should excrete at least 50 mg. per 10 stone weight for the 2½-hour period on the second day, while an inadequate vitamin C intake is manifested by requiring a longer period of time before obtaining a satisfactory response to the test dose.

Other workers have recommended a 24-hour saturation test. Also, for this purpose, ascorbic acid may be used intravenously. According to Hawley and Stephens,<sup>10</sup> in saturated subjects the maximum excretion, after oral administration, is obtained during the third, fourth, fifth, and sixth hours; while after intravenous use it occurs during the first and second hours.

#### METHOD OF STUDY

In this study the subjects were requested to avoid eating fruit, fruit juices, and those foods high in vitamin C, immediately before and during the test. After breakfast, the bladder was emptied, and the subject was given 200 mg. ascorbic acid, and a glass of water. (The tablets were swallowed in the presence of the author.) The bladder was emptied every hour for six hours. The majority of the urine specimens were titrated\* (with a standardized, purified sodium 2,6 dichlorophenol indophenol) within fifteen to twenty minutes after voiding. In one part of this study the specimens were collected over a 24-hour period. The night specimens were preserved with acetic acid and titrated the following morning.

All were adults; the majority were between 20 to 30 years of age.

One group of seven women and eight men, presenting a history of having had considerable fresh fruits and vegetables for at least one month prior to the test, were selected for the first part of this study. These were considered to be average normal adults on an adequate vitamin C diet. A second group of eighteen men, presenting the history of having had a limited quantity of fruit and fresh vegetables, were selected on the assumption that they had an inadequate vitamin C diet. The third part of the investigation consisted of four patients suspected of a definite vitamin C deficiency. An additional study was undertaken on three men to determine the excretion of ascorbic acid during a twenty-four hour period, without and with the test dose.

#### RESULTS

Fifteen adults, considered to have had an adequate vitamin C diet, received an oral administration of 200 mg. of ascorbic acid (Table I). The average total excretion for the four-hour period was 27 mg.; the total for the six-hour period was 50 mg. The range for the four-hour test was 17.3 to 71.6 mg., while the six-hour range was from 27.9 to 82.3 mg.

\*Ascorbic acid, which is a strong reducing agent, is capable of decolorizing sodium 2,6 dichlorophenol indophenol. It is well understood that other substances may also be present in urine possessing this property. These substances can be removed with mercuric or barium acetate. For clinical purposes, this does not appear to be necessary.

TABLE I

ADEQUATE VITAMIN C INTAKE SHOWING MILLIGRAMS EXCRETED IN URINE\*

MILLIGRAMS PER HOUR								
	FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR	TOTAL MG. 4 HOURS	TOTAL MG. 6 HOURS
(Group 1—Females)								
1.	5.6	4.2	20.3	9.7	12.7	8.8	39.8	61.3
2.	5.5	1.4	7.0	9.0	27.2	19.3	22.9	69.4
3.	3.6	0.0	10.2	9.9	20.7	23.0	23.7	67.4
4.	5.1	14.8	47.0	4.7	5.6	5.1	71.6	82.3
5.	6.4	2.1	5.9	9.9	15.5	7.7	24.3	47.5
6.	4.2	2.5	3.4	7.2	5.9	4.7	17.3	27.9
7.	1.0	1.6	5.6	12.1	23.0	9.4	20.3	52.7
Ave.	4.49	3.80	14.20	8.93	15.80	11.14	31.41	58.36
(Group 2—Males)								
8.	0.4	6.7	6.1	11.2	9.8	7.3	24.4	41.5
9.	1.6	7.9	3.6	6.2	7.8	7.7	19.3	34.8
10.	1.4	9.4	19.0	7.2	8.2	25.2	37.0	70.4
11.	2.5		18.2	7.2	6.9	13.2	27.9	48.0
12.	1.3	5.5	7.6	6.7	8.1	5.1	21.1	34.3
13.	0.5	0.9	8.5	13.7	7.1	4.8	23.6	35.5
14.	0.5	2.8	9.7	9.8	8.0	7.2	22.8	38.0
15.	0.3	0.4	3.6	16.3	17.7	6.8	20.6	45.1
Ave.	1.07	4.20	9.53	9.79	9.2	9.7	24.59	43.49
(Averages of Groups 1 and 2)								
	2.56	4.00	11.57	9.41	12.9	10.33	27.54	49.96

\*After oral administration of a 200 mg. test of Ascorbic Acid.

The eighteen subjects selected, because their history indicated an inadequate vitamin C diet, excreted, after oral administration of 200 mg. of ascorbic acid, an average of 3.2 mg. for four hours and 5.1 mg. for six hours (Table II).

TABLE II

INADEQUATE VITAMIN C INTAKE SHOWING MILLIGRAMS EXCRETED IN URINE\*

	MILLIGRAMS PER HOUR						TOTAL MG. 4 HOURS	TOTAL MG. 6 HOURS
	FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR		
16.	2.1	0.9	0.5	0.4	2.2	0.5	3.9	6.6
17.	0.0	1.0	0.5	0.0	0.3	1.3	1.5	3.1
18.	1.2	0.9	0.0	1.2	0.3	0.9	3.3	4.5
19.	0.5	0.6	1.4	1.0	0.8	1.0	3.5	5.3
20.	0.7	0.4	0.0	1.4	1.1	0.4	2.5	4.0
21.	1.8	0.5	0.4	0.6	1.0	0.5	3.3	4.8
22.	1.0	0.5	0.5	0.5	0.8	0.6	2.5	3.9
23.	0.4	0.6	0.4	0.4	0.6	0.8	1.8	3.2
24.	1.4	0.6	0.8	0.3	0.6	0.6	3.1	4.3
25.	1.0	0.5	0.4	0.7	0.6	0.5	2.6	3.7
26.	0.4	2.5	2.5	0.7	1.0	2.4	6.1	9.5
28.	0.1	0.2	0.5	0.9	0.9	1.2	1.7	3.8
29.	0.2	0.5	0.9	0.8	1.0	1.1	2.4	4.5
30.	0.9	1.0	2.2	0.9	1.9	1.4	5.0	8.3
31.	1.0	1.1	2.8	0.3	1.2	1.7	5.2	8.1
32.	0.5	0.5	1.1	1.1	1.2	0.9	3.2	5.3
33.	0.6	0.7	1.6	0.5	1.0	0.8	3.4	5.2
34.	0.8	0.6	0.6	0.5	0.8	0.9	2.5	4.2
Ave.	0.81	0.76	0.95	0.63	0.96	0.97	3.20	5.13

\*After oral administration of 200 mg. of Ascorbic Acid.

The four patients suspected of a vitamin C deficiency were tested with this saturation test (Table III). During the six-hour period, the average excretion was 6.55 mg. After receiving approximately 200 mg. of vitamin C (from fruit



juices) daily for sixteen days, these patients were retested. The average excretion for the 6-hour test period was 118.3 mg. Three of the four patients excreted 116.9, 163.5, and 149 mg. of ascorbic acid, indicating that their tissues were well saturated.

TABLE III  
RESPONSE TO DAILY ORAL ADMINISTRATION OF VITAMIN C SHOWING  
MILLIGRAMS EXCRETED IN URINE\*

PATIENT	TEST	MILLIGRAMS PER HOUR						TOTAL MG. 4 HOURS	MG. 6 TOTAL HOURS
		FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR		
29	1st	0.2	0.5	0.9	0.8	1.0	1.1	2.4	4.5
	2nd†	0.3	4.7	28.6	29.7	37.1	16.5	63.0	116.9
30	1st	0.9	1.0	2.2	0.9	1.9	1.4	5.0	8.3
	2nd†	3.0	22.2	31.5	31.1	32.0	43.7	87.8	163.5
31	1st	1.0	1.1	2.8	0.3	1.2	1.7	5.2	8.1
	2nd†	1.1	8.6	31.0	21.0	36.8	50.5	61.7	149.0
32	1st	0.5	0.5	1.1	1.1	1.2	0.9	3.2	5.3
	2nd†	0.2	0.4	7.4	15.5	10.2	10.2	23.5	43.9

\*After oral administration (test dose) of 200 mg.

†Second test was made 16 days after the first test; during this interval the patients received approximately 200 mg. of vitamin "C" daily.

TABLE IV  
TWENTY-FOUR HOUR URINARY EXCRETION OF VITAMIN C BEFORE AND AFTER ORAL  
ADMINISTRATION OF ASCORBIC ACID  
Showing milligrams per specimen

TIME HOURS	SUBJECT 8		SUBJECT 13		SUBJECT 15	
	BEFORE	AFTER*	BEFORE	AFTER*	BEFORE	AFTER*
1	0.2	1.6	0.2	0.5	0.1	0.3
2	0.4	7.9	0.1	0.9	0.02	0.4
3	†	3.6	†	8.5	0.1	3.6
4	0.5	6.2	0.2	13.7	0.2	16.3
5	2.1	7.8	†	7.1	0.7	17.7
6	1.3	7.7	1.2	4.8	0.4	6.8
8	†	6.5	0.8	3.6	0.2	5.5
10	0.6	3.7	0.5	5.0	1.0	0.8
12	1.4	12.4	0.8	1.8	0.5	0.3
14	0.4	4.2	†	†	†	†
20	†	†	0.0	5.3	†	3.7
22	7.5	4.9	†	†	†	†
24	3.1	6.5	0.9	1.6	0.5	0.6
Total	17.4	73.0	4.7	52.8	3.7	55.9

\*After oral administration of 200 mg. of Ascorbic Acid.

†No urine specimen collected.

Another individual with a history of a very inadequate vitamin C diet excreted 3.8 mg. in the 6-hour period. After receiving 50 mg. of ascorbic acid daily for six days, a second test resulted in an excretion of 3.7 mg. After twenty-nine days on this 50 mg. dose, the response to a test dose was 10.3 mg. for the six-hour period. The daily dose was increased to 75 mg. Six days later the response to the test dose produced satisfactory results, with an excretion of 47 mg. in six hours.

Finally, a study was made on three individuals on an adequate vitamin C diet to determine the rate of ascorbic acid excretion during two consecutive twenty-four hour periods (Table IV). During the first twenty-four hour period these men had a normal diet without the test dose. After the last urine specimen

was collected for this first period, these men were given 200 mg. of ascorbic acid, and the urine specimens were collected for the second twenty-four-hour period. The results show that there is a marked difference in the ascorbic acid content of the 24-hour urine specimens before and after a test dose is administered.

#### SUMMARY

1. Satisfactory results can be obtained with a 200 mg. saturation test.
2. Since the maximum rate of excretion, after oral administration, occurs during the third, fourth, fifth, and six hours, a six-hour saturation test appears to be satisfactory. A four-hour saturation test probably will give satisfactory results. In either case, hourly specimens are not necessary.
3. An adequate vitamin C diet is indicated by a urinary excretion of approximately 20 mg. or more of ascorbic acid in four hours, or approximately 30 mg. or more in six hours. Less than 10 mg. in six hours can be definitely considered inadequate.

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## METHODS FOR THE DETERMINATION OF THIOURACIL IN TISSUES AND BODY FLUIDS\*

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DURING the past two years the goitrogenic effects of certain thioureas and sulfonamides have been reported by several observers.<sup>1-7</sup> Mackenzie, Mackenzie, and McCollum<sup>1</sup> noted, while studying the effect of sulfaguanidine on the bacterial synthesis of vitamins in rat intestines, that marked enlargement of the thyroid resulted from this drug. Richter and Clisby,<sup>2</sup> when investigating the effects of various unpleasant tasting substances in rats, found that phenyl thiourea induced goiter formation. Kennedy and Purves<sup>3</sup> demonstrated in rats a goitrogenic effect of rapeseed, this effect being ascribed to thiourea.<sup>4</sup> Subsequent studies by Mackenzie and Mackenzie,<sup>5</sup> as well as by Astwood and associates,<sup>6</sup> revealed that a large number of sulfonamides and thioureas caused in rats not only marked thyroid enlargement, but also a decrease in the basal oxygen consumption. Whereas these changes were prevented by hypophysectomy or by the simultaneous administration of thyroxine; diiodotyrosine and potassium iodide had no effect. These sulfonamides and thioureas inhibit the production of the thyroid hormone, leading to a fall in the basal metabolism and enlargement of the thyroid gland. Astwood<sup>7</sup> found that 2-thiouracil possessed these properties to a greater degree than any of a large number of substances which he tested. He also reported<sup>8</sup> that three thyrotoxic patients treated with thiourea or thiouracil experienced a fall in the basal oxygen consumption and a remission of their symptoms. Williams and Bissell<sup>9</sup> confirmed these observations and also found that the plasma protein-bound iodine returned to normal in association with thiouracil therapy. An arbitrary dosage of about one gram daily has been used, but it is desirable to ascertain the minimal effective dosage since one case of severe agranulocytosis resulted from the thiouracil treatment.<sup>8</sup> The ultimate solution to this problem must be based upon the response of the patient to varying dosages, but it would be very helpful to know about the rate of absorption from the gastrointestinal tract, the distribution throughout the body, and the rate of excretion of the drug. Several months ago we began an investigation of these points, but since no methods had been reported for the determination of thiouracil in tissues and body fluids, it was first necessary to attack this problem. In this paper we are presenting the methods which we have found to be satisfactory.

The methods chosen are based on Grote's<sup>10</sup> observation that a blue color was formed by substances of a  $C \equiv S$  type when treated with a special reagent.

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Grote's reagent was produced by the treatment of sodium nitroferrieyanide in sodium bicarbonate solution with hydroxylamine hydrochloride followed by bromine. The reaction is apparently limited to compounds of divalent sulfur linked to a single nonmetallic element; thiouracil (2-thio, 6-oxypyrimidine) has this linkage.

#### METHODS

*A. Preparation of Grote's Reagent.*—In preparing the reagent used by Grote, 0.5 Gm. of sodium nitroferrieyanide is dissolved in 10 c.c. of water in a small beaker at room temperature. To this mixture is added 0.5 Gm. of hydroxylamine hydrochloride and then 1 gram of finely powdered sodium bicarbonate. The beaker is kept covered with a watch glass until the evolution of gas has ceased and then 0.1 c.c. of bromine is added. The cover is replaced until gas no longer evolves. This solution, which is clear mahogany brown, is filtered into a 25 c.c. volumetric flask and then diluted to volume. Before using this solution it is further diluted 1:5.<sup>11</sup>

*B. Determination of a Standard Curve.*—A 10 mg. per cent aqueous solution of thiouracil\* is prepared. The pH is adjusted to 8.5 with normal sodium hydroxide, using a glass electrode for the pH determinations. Aliquots of 0.05 to 1 c.c. are diluted with distilled water to 1 c.c. To each tube is added 1 c.c. of Grote's reagent. After standing for fifteen minutes, 4 c.c. of 1 per cent sodium chloride is added to each tube to a total volume of 6 c.c., and the intensity of the color is immediately determined in the Evelyn photoelectric colorimeter, using a 660 filter and No. 6 diaphragm. The tube used as a blank contains 1 c.c. of Grote's reagent and 5 c.c. of one per cent sodium chloride. When the results are plotted on semilogarithmic paper, a straight line is obtained.

*C. Urine Analysis.*—The urine is preserved with chloroform or hydrochloric acid. The pH of the entire specimen is adjusted to from 8.5 to 9 with normal sodium hydroxide. About 10 c.c. of the urine are centrifuged and from 0.25 to 0.5 c.c. of the clear layer is pipetted into a colorimeter tube. Following the addition of 1 c.c. of Grote's reagent, the tube is allowed to stand for fifteen minutes. It is then diluted with 1 per cent sodium chloride to 6 c.c. and read immediately in the colorimeter, as in the case of the standard solutions.

*D. Blood Analysis.*—To 1 c.c. of oxalated blood is added 1 c.c. of distilled water. The tube is shaken and the pH is adjusted to from 8 to 8.5. Then 0.1 c.c. of a 5 per cent solution of trypsin (technical) is added, and the mixture is incubated at 37° C. for twelve hours. A protein filtrate is prepared, using 1 c.c. of the digested blood mixture, 7 c.c. of distilled water, 1 c.c. of 5 per cent copper sulfate, and 1 c.c. of 7 per cent sodium tungstate. The pH of the filtrate is adjusted to from 8.5 to 9. Then 5 c.c. of the filtrate are pipetted into a colorimeter tube, and 1 c.c. of Grote's reagent is added. After fifteen minutes, the amount of color development is estimated in the colorimeter as in the case of the standard solutions. Occasionally a slight cloudiness develops after the addition of Grote's reagent, in which case the solution is filtered through No. 2 Whatman filter paper.

\*The thiouracil was supplied by the Lederle Laboratories, Inc., Pearl River, N. Y.

**E. Tissue Analysis.**—To the tissue is added an equal volume of water and about one-half this volume of normal sodium hydroxide. A homogenous suspension is prepared in a Waring Blender or in a mortar. The mixture is allowed to stand overnight in the icebox, and an aliquot of 1 c.c. is taken for analysis. A protein filtrate is prepared by means of the copper sulfate-sodium tungstate mixture, as in the case of the blood. Following the adjustment of the pH from 8.5 to 9.0, 5 c.c. of the filtrate are pipetted into a colorimeter tube. Then 1 c.c. of Grote's reagent is added, and the color is estimated after fifteen minutes.

#### COMMENTS

**A. Physical and Chemical Properties of Thiouracil.**—Little or no data on the properties of thiouracil seem to have been published since Wheeler and Liddle<sup>12</sup> reported the synthesis of this compound in 1908. Thiouracil is a white, odorless, bitter powder. It is insoluble in methyl alcohol, ethyl alcohol, carbon tetrachloride, acetone, and mineral acids; it can be partially extracted from an acidified aqueous solution by shaking with ether over a prolonged period of time. A saturated aqueous solution contains about 50 mg. of thiouracil per 100 c.c. at 25° C. Due to its acid properties, it forms very soluble salts on addition of sodium or potassium hydroxide to an aqueous suspension. Solutions of thiouracil are not affected by heating to 80° C. for ten minutes, although they lose 20 per cent of their thiouracil content when kept in a boiling water bath for five minutes; 100 per cent when autoclaved for five minutes at 105° C. under fifteen pounds of pressure.

Dry thiouracil is stable for months at room temperature. It has been found to remain stable for several days when kept in the icebox (5° C.) whether in the form of an aqueous solution, or in urine, blood, or tissue extracts. No destruction occurred when a solution containing 20 mg. per 100 c.c. of thiouracil was exposed to direct sunlight for one hour.

**B. Factors Affecting the Color Reaction.**—The maximal color reaction between thiouracil and Grote's reagent takes place at a pH from 8 to 9. The further the pH is removed from this range, the less intense is the color produced.

Although some color begins to develop as soon as Grote's reagent is added to a solution of thiouracil, the maximal reaction, at room temperature, requires about fifteen minutes. If an interval longer than fifteen minutes is allowed to elapse between the addition of the reagent to the solution of thiouracil and the dilution of this mixture to 6 c.c., the color so produced gradually decreases; after seventy-five minutes, the intensity is only one-half that obtained after fifteen minutes (Fig. 1). On the other hand, the color of the diluted mixture decreases only 3 per cent on standing at room temperature for sixty minutes. The rate of the reaction can be increased by heat; the maximal color at 55° C., 60° C., and 75° C. is obtained in five, four, and two minutes respectively. However, the process of heating may cause some cloudiness which interferes with colorimetric estimations. Light was shown to affect the reaction, since only 40 per cent of the usual color developed when 0.5 c.c. of a 20 mg. per 100 c.c. solution of thiouracil was added to Grote's reagent and permitted to remain in the dark for fifteen minutes.

Undiluted Grote's reagent will remain stable for two weeks or more if kept in the icebox. However, it should be tested with a standard solution of thiouracil at intervals of two to three weeks. The only component of Grote's reagent which we have thus far observed to deteriorate is hydroxylamine hydrochloride. We have found this substance to lose appreciable activity within six weeks, in spite of keeping it stoppered and in the icebox most of the time.

Although a number of factors affect the color intensity, when the determinations are performed in the manner outlined previously, relatively constant results are obtained, making it unnecessary to make frequent estimations on standard solutions.

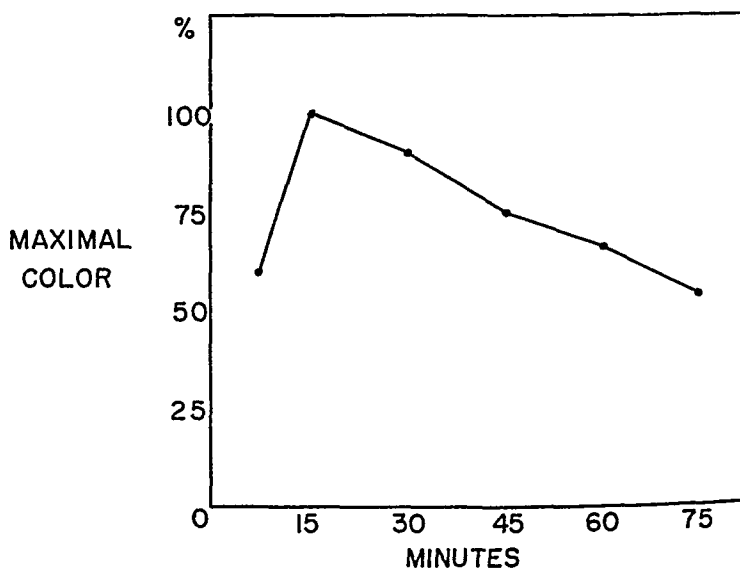


Fig. 1.—The effect of time on the intensity of the color produced by the reaction at room temperature of thiouracil (8 mg. per cent solution) with Grote's reagent.

*C. Analysis of Specimens.*—In all of the analyses conducted we have used as a blank for the colorimeter readings 5 c.c. of distilled water and 1 c.c. of Grote's reagent. This is permissible because all of the specimens to be assayed, except urine, give a clear colorless filtrate, and the quantity of urine used is so small that the yellow color does not interfere with the determination. (1) *Urine.*—Thiouracil is soluble in urine in dilutions of 1:2000, and, therefore, it should be in solution with the dosages ordinarily used therapeutically. When the pH of the urine specimen is adjusted to 8.5, complete solubility is assured. A slight precipitate usually follows alkalinization, but this can be removed by centrifugation. (2) *Blood.*—The analysis of blood proved to be a much more difficult problem than that of urine. It is necessary to obtain a clear filtrate for accurate colorimetric estimations. However, it is found that a large part of thiouracil remains in the precipitate. Zinc sulfate, sodium hydroxide mixture, trichloroacetic acid, and tungstic acid all proved to be unsatisfactory as protein precipitants because they render the thiouracil insoluble. Since the concentration of thiouracil in the resulting filtrates is always far below that necessary

to produce a saturated solution, i.e., about 50 mg. per 100 c.c., it seems that thiouracil at an acid pH combines with the precipitated proteins or their breakdown products. This is borne out by the fact that when the proteins of hemolyzed blood were digested with trypsin, recoveries were incomplete when the above precipitants were used. Precipitation with sodium tungstate and copper sulfate, on the other hand, yielded satisfactory recoveries. Thus, the first step in the analysis of blood is to hemolyze it with distilled water. The pH is then adjusted to from 8 to 8.5, since this is the range for maximal activity of trypsin. Incubation is carried out at 37° C. for at least eight hours; shorter intervals lead to incomplete extraction. As the blood becomes more alkaline upon incubation, the protein filtrate is sometimes blue in color due to copper. However, upon reducing the pH to 8.5, the filtrate again becomes colorless. Occasionally, when Grote's reagent is added to the filtrate, a cloudiness develops but this is readily removed by filtration. (3) *Tissues*.—When the quantity of thiouracil is expected to be small, the amount of fluid added should be only enough to permit the formation of a thin emulsion of tissue. Sodium hydroxide is used to extract thiouracil from the tissues; for complete extraction it is advisable to permit the mixture to stand overnight. It is necessary to prepare a protein-free filtrate in order to obtain a colorless solution. Digestion of the tissue proteins with trypsin has been found unnecessary.

TABLE I  
RECOVERY OF THIOURACIL, ADDED TO THE URINE AND BLOOD

URINE			BLOOD		
ADDED MG./100 C.C.	RECOVERED MG./100 C.C.	PER CENT RECOVERY	ADDED MG./100 C.C.	RECOVERED MG./100 C.C.	PER CENT RECOVERY
0	0	---	0	0	---
2	2	100	0.4	0.32	80
4	3.9	98	1.6	1.4	88
5	5	100	2.4	2.4	100
6	6	100	3.2	3.2	100
8	8	100	4.0	4.0	100
10	10	100	6.0	5.9	98
20	19.8	99	8.0	8.0	100
50	49.2	98	10.0	9.6	96
100	96	96			

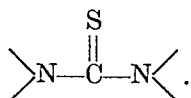
The analysis of liver differs slightly from that of the other tissues in that it is advisable to remove the excess fat. A suspension of liver in a weakly alkaline solution is extracted with ether, preferably overnight. The ether is then decanted and the usual procedure is resumed.

*D. Recoveries*.—In an accompanying paper<sup>13</sup> are presented the results of numerous analyses of the thiouracil content of urine, blood, and tissues of patients. In a number of experiments, thiouracil was added to such materials in quantities sufficient to yield concentrations approximating those obtained in the analyses. Assays of the content of thiouracil in these specimens usually gave values which were from 90 to 100 per cent of the amount added. Very small quantities of thiouracil could be recovered to an extent of not less than around 80 per cent due to analytical error in such low ranges of concentration. The results given in Tables I and II are representative of the ones which we have obtained repeatedly.

TABLE II  
RECOVERY OF THIOURACIL ADDED TO TISSUE SUSPENSIONS

ADDED MG./100 C.C.	LIVER		KIDNEY		MUSCLE	
	RECOVERED MG./100 C.C.	PER CENT RECOVERY	RECOVERED MG./100 C.C.	PER CENT RECOVERY	RECOVERED MG./100 C.C.	PER CENT RECOVERY
0	0	---	0	---	0	---
0.4	0.4	100	0.3	75	0.4	100
0.8	0.7	87	0.7	87	0.7	87
1.2	1.2	100	1.3	107	1.2	100
1.6	1.5	94	1.6	100	1.5	94
2.0	2.0	100	2.0	100	2.0	100
4.0	3.8	95	3.8	95	3.7	93
6.0	6.0	100	6.1	101	5.9	98
8.0	7.6	95	7.6	95	7.8	97
10.0	9.6	96	9.8	98	10.0	100
20.0	19.8	99	19.9	99	20.0	100
40.0	38.0	96	39.0	97	38.0	96
60.0	55.0	91	56.0	93	58.0	96
100.0	98.0	98	98.0	98	100.0	100

E. *Evidence for the Identity of Thiouracil in Tissues and Body Fluids.*—To test the specificity of our colorimetric procedure, a great number of possible breakdown products of thiouracil, as well as other types of sulfur compounds, were treated with Grote's reagent. These experiments indicate that an appreciable color with the reagent, under our conditions, is produced only by those possessing the group



Potassium sulfide, sodium sulfite, thiocyanate, and thiosulfate, uracil, urea, creatinine, cysteine, cystine, glutathione, choline, methionine, thiamine, and sulfanilamide were tested in concentrations equivalent to 100 mg. per 100 c.c. of thiouracil. According to Grote's findings,<sup>10</sup> all the compounds in this list which contain divalent sulfur linked to a single nonmetallic element can be expected to yield a color with the reagent. This agrees with our findings that sodium thiocyanate and thiosulfate, in neutral or acid solution, produce a blue color with the reagent; even creatinine will react in this way in acid solution. However, none of the above compounds yielded any color when their solutions were adjusted to a pH from 8.5 to 9 before addition of Grote's reagent, nor did their presence inhibit or enhance the color formation due to 10 mg. per 100 c.c. of thiouracil. Sodium pentothal yielded 10 per cent, and thiobarbituric acid 35 per cent of the color produced by an equivalent amount of thiouracil.

Thiourea, under the above conditions, yields an intense color with Grote's reagent; this color is a pure blue as contrasted with a greenish-blue obtained with thiouracil. Rough absorption spectra of the color of thiouracil and thiourea with the reagent were obtained by determining the absorption of such solutions in the Evelyn photoelectric colorimeter with the filters available in our laboratory; the filters used had their maximum transmission at 420, 440, 520, 540, 565, 620, and 660 m $\mu$ . At the same time, similar absorption curves were obtained for urines collected after administration of either thiouracil or thiourea by mouth, with due corrections for the light absorption of the urine and of



Grote's reagent. Fig. 2 shows that there is considerable difference in the absorption curves of the compound formed by Grote's reagent with thiourea and thiouracil, respectively. This was to be expected from the visually apparent difference in color between these compounds referred to earlier.\* However, a marked similarity is obvious between the shape of the absorption curve of pure thiouracil or thiourea and of the urines containing the excretion products of either one.† At wave lengths up to 520 mμ, urines treated with Grote's reagent, when corrected for the absorptions of the reagent and the urine, showed some absorption; the same was found for urines devoid of thiouracil or thiourea. Obviously Grote's reagent reacts with some normal urinary constituent to yield a compound which absorbs in this region. Neither the urines nor the reagent showed any appreciable absorption at wave lengths greater than 520 mμ.

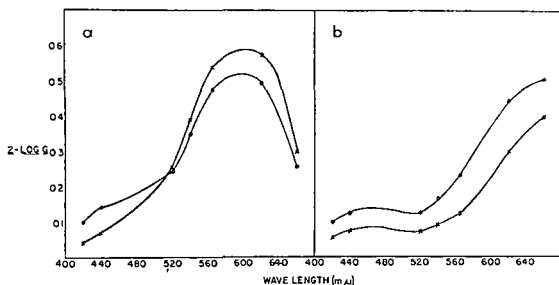


Fig. 2.—a, Absorption curves of the reaction product of Grote's reagent with thiourea (x — x) and with urine containing the excretion product of orally administered thiourea (o — o).

b, Absorption curves of the reaction product of Grote's reagent with thiouracil (x — x) and with urine containing the excretion product of orally administered thiouracil (o — o).

In several instances, extracts were prepared of liver, heart muscle, and skeletal muscle of guinea pigs which had not been given any thiouracil. These extracts, as well as urines and blood filtrates from patients who had not been treated with thiouracil, never yielded any color when Grote's reagent was added to them.

#### SUMMARY

1. Methods are described for the estimation of the content of thiouracil in tissues, urine, and blood.

2. The methods depend upon the fact that the  $\text{>N}-\overset{\text{S}}{\underset{\parallel}{\text{C}}}-\text{N}<$  linkage of thi-

\*The validity of the above approximate spectrometric method was established by comparing the absorption curves of Fig. 2 with more complete ones obtained for thiouracil with a Hardy recording spectrophotometer, and for thiourea with a Beckmann spectrophotometer. The two sets of spectra for the same compound were superimposable. Moreover, by extending the spectrum of thiouracil to 700 mμ, it was found to show a maximum at about 630 mμ, thus making it appear very similar to that of thiourea except for a shift of about 90 to 100 mμ towards the red part of the spectrum.

†The differences in the absolute magnitudes of absorption, as opposed to the relative absorptions expressed by the shapes of the curves, between the pure drugs and their urinary excretion products is due, of course, to differences in the concentration of the chromogen in these respective solutions.

ouracil reacts with sodium aquoferrieyanide in alkaline solution giving a greenish color which can be quantitated in a colorimeter.

3. Thiouracil in blood is partially bound to proteins. It can be liberated by tryptic digestion before assaying.

4. Using the methods which we have described, almost complete recovery of added thiouracil can be obtained.

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# THE PREPARATION OF A MORE STABLE CONCENTRATED UREASE SOLUTION\*

CAPT. GEORGE R. KINGSLEY, SN. C., A.U.S.

UREASE solutions prepared by extraction of Jack Bean Meal with alcohol and water are not stable at room temperature and when refrigerated may not be kept safely for more than one month. The alcohol in these extracts may also decrease the stability of the color developed after nesslerization. Water solutions of commercial urease powder are also unstable. Urease powder cannot be conveniently measured uniformly unless weighed; this is a time-consuming procedure.

A simple procedure is proposed for the preparation of concentrated solutions of extracts of Jack Bean Meal and of commercial urease which are stable several weeks at room temperature.

TABLE I

COMPARISON OF FRESHLY PREPARED UREASE, AGED UREASE, AND AGED JACK BEAN MEAL EXTRACT SALT SOLUTIONS IN THE DETERMINATION OF BLOOD UREA NITROGEN

UREASE		JACK BEAN MEAL EXTRACT
FRESH	AGED 17 WK. AT 25° C.	AGED 9 WK. AT 25° C.
MG. PER CENT	MG. PER CENT	MG. PER CENT
11.6	12.4	11.8
10.6	12.3	12.4
10.3	11.3	11.8
10.6	11.5	11.0
16.7	17.7	16.7
10.2	11.1	10.2
11.2	10.8	11.0
14.3	13.9	14.0
13.1	12.8	13.0
13.9	14.2	14.3
12.9	13.2	13.0
15.8	15.6	15.5
12.8	13.2	13.5
18.8	18.8	19.0
18.2	17.9	17.8
13.2	13.0	12.8
9.1	9.2	9.2
20.8	21.2	21.0
21.2	21.4	21.5
17.8	17.5	17.3
13.7	14.0	13.6
57.0	58.0	56.0
17.8	17.7	17.9
12.5	12.7	12.8
17.0	17.3	17.5
73.5	76.0	73.0
19.7	20.2	19.3
16.9	16.2	16.5
11.5	12.0	11.5
18.0 av.	18.4 av.	18.1 av.

\*From the Laboratories, McCloskey General Hospital, Temple, Texas.  
Received for publication, Jan. 29, 1914.

## PROCEDURE

1. *Preparation of Extract of Jack Bean Meal.*—To 150 c.c. of saturated sodium chloride solution in a 300 c.c. Erlenmeyer flask, add 40 Gm. of Jack Bean Meal, 4 Gm. of permutit, and shake vigorously several minutes. Let the mixture stand twenty-four hours at room temperature. Shake the mixture again a few minutes and then centrifuge. Filter the supernatant solution through ordinary filter paper. Cover filter, as the filtration may require several hours. Keep in a small dropper bottle. Use 1 drop for each 2.5 c.c. of acid tungstate protein free filtrate.

2. *Preparation of Commercial Urease Solution.*—To 50 c.c. of saturated sodium chloride solution, add 2 Gm. of urease (Squibb double strength powder) and shake vigorously a few minutes. Let stand one hour and filter. Cover filter, as this filtration may require a few hours. Keep in bottle with dropper. Use 1 drop of this solution for each 2.5 c.c. of acid tungstate protein-free filtrate.

Ordinary precautions against contamination of the urease solutions should be taken.

The stability of the saturated salt extracts aged from nine to seventeen weeks at room temperature is indicated by the good agreement between old and fresh extracts (Table I). Blank determinations made upon fresh saturated salt extracts and 9 to 17 weeks' old saturated salt extracts used in the preparation of the data in Table I were negative for ammonia nitrogen or ammonia nitrogen-forming elements. The blood urea nitrogen method of Karr<sup>1</sup> was used for the comparison of the extracts. Blood specimens for the data in Table I were taken from hospital patients during routine laboratory examination.

## SUMMARY

A procedure is described for the preparation of concentrated extracts of Jack Bean Meal and of commercial urease which are stable several weeks at room temperature.

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## *CLINICAL AND EXPERIMENTAL*

### SHOCK RESULTING FROM THE INTRAPERITONEAL IMPLANTATION OF RECONSTITUTED DESICCATED MUSCLE\*

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THE intraperitoneal implantation of skeletal muscle pulp has been demonstrated by Moon<sup>1</sup> to give rise to fatal shock when appropriate doses are used. We have modified this technique by desiccating the ground muscle from the frozen state and subsequently introducing the dry muscle plus a small volume of water into the peritoneal cavity by means of a syringe and trocar.

#### METHODS

Healthy mongrel dogs under nembutal anesthesia (1 c.c. per 5 pounds body weight) were used. Skeletal muscle was obtained from the hind limb of a normal dog with the usual sterile surgical precautions. The muscle was passed through a sterile meat grinder and desiccated from the frozen state by the adtervac process.<sup>2</sup> This procedure yielded a coarse, partly fibrous powder able to soak water readily. The dried muscle, 20 grams per unit, was stored in Erlenmeyer flasks for several weeks. Prior to use, 25 c.c. water were added to each unit. The resulting thick paste of muscle was introduced under pressure into the peritoneal cavity by means of a sterile metal syringe and a large trocar ordinarily used for suprapubic stab. The small incision for the trochar was closed with two to three sutures. The animals were observed periodically, and small samples (2 to 3 c.c.) of venous blood were obtained for the following procedures: hemoglobin concentration of blood (photoelectric),<sup>3</sup> red blood cell count, hematocrit,<sup>4</sup> whole blood- and plasma-specific gravity.<sup>5</sup> As soon as the animals were completely awake from the anesthesia, water by mouth was allowed.

\*From the William Buchanan Blood, Plasma, and Serum Center.  
Received for publication, Aug. 16, 1943.

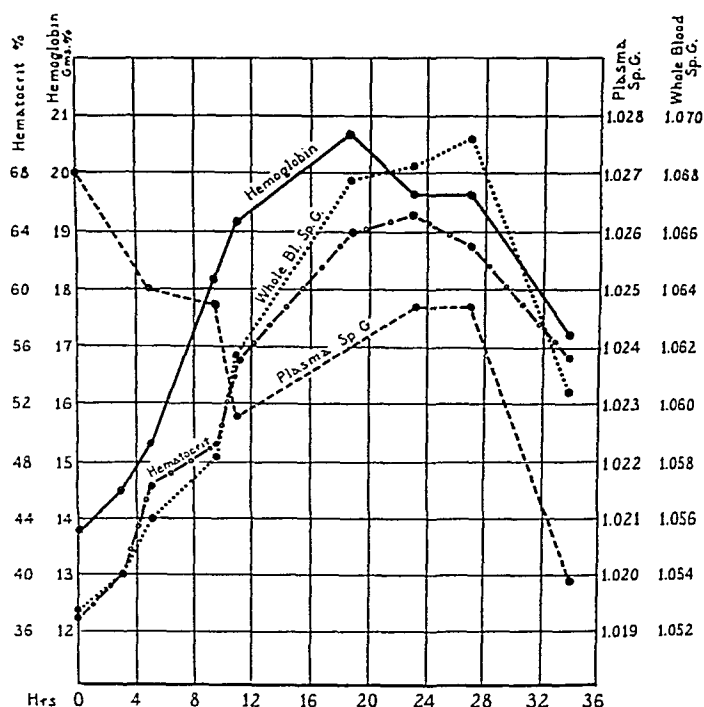


Fig. 1.—Fatal shock by intraperitoneal implantation of muscle substance. There is a terminal hemodilution in this case. The plasma specific gravity was steadily lowered.

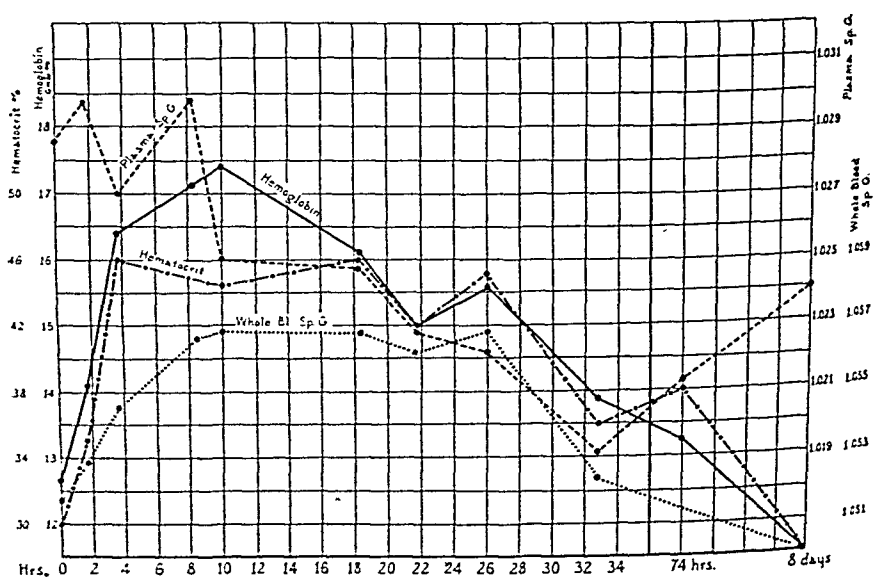


Fig. 2.—Recovery following a period of hemoconcentration. A prominent anemia developed after recovery, but the plasma specific gravity was elevated.

In the seven experiments conducted, five animals received 15 to 25 grams of desiccated muscle, and two received 40 grams.

Specimens of tissue from the viscera for microscopic study (H and E stain) were obtained from the animals after death.

#### RESULTS

All animals recovered from the anesthetic within 5 to 10 hours and were able to stand up in their cages. Three animals subsequently died in the state of shock; but four animals survived after a period of hemoconcentration and "toxic appearance" and clinically appeared normal for the one to several weeks observed.

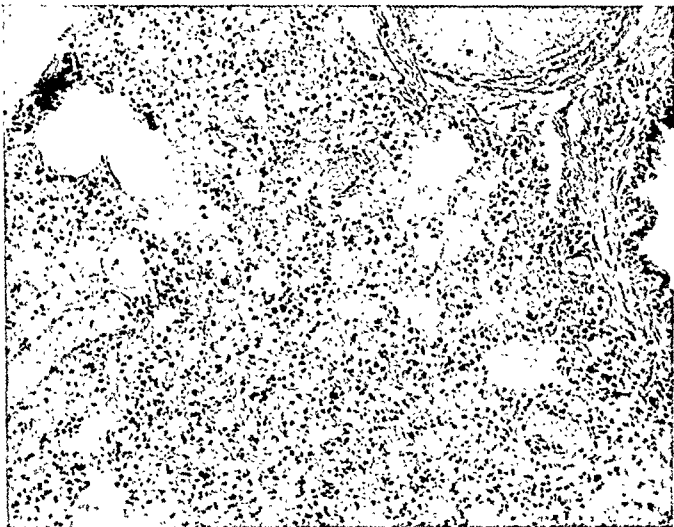


Fig. 3.—Photomicrograph of lung following fatal shock ( $\times 225$ ). There is marked capillarto-venous stasis, edema, and capillary hemorrhages.

All animals displayed venous hemoconcentration. The animals that expired in shock exhibited the greatest peak of hemoconcentration from 18 to 22 hours after the implantation, while of those that survived, the peak was reached in 8 hours in two, and in 18 to 22 hours in three. The degree of hemoconcentration was much lower in those animals that survived when compared to the group that expired. In the survival group, hemoconcentration began to lessen after 10 to 22 hours and reached near-control levels within 30 to 48 hours of the implantation, and in the subsequent days anemia developed. Two of the animals that died displayed a moderate terminal decrease in the venous hemoconcentration.

The plasma-specific gravity was lowered substantially before the animals were allowed water. This decrease began shortly after the implantation and continued for as long as 34 hours. According to Weech's formula,<sup>6</sup> such decrease in plasma-specific gravity represents a lowering of the plasma protein concentration. With survival, the plasma-specific gravity was elevated as anemia appeared.

When the animals were fully awake from the anesthetic, a ravenous thirst was observed. Vomiting was prominent, but some water appeared to be retained. There was weakness and a lethargic appearance throughout the experimental period, and the dogs surviving were disinterested in food for 48 to 72 hours.

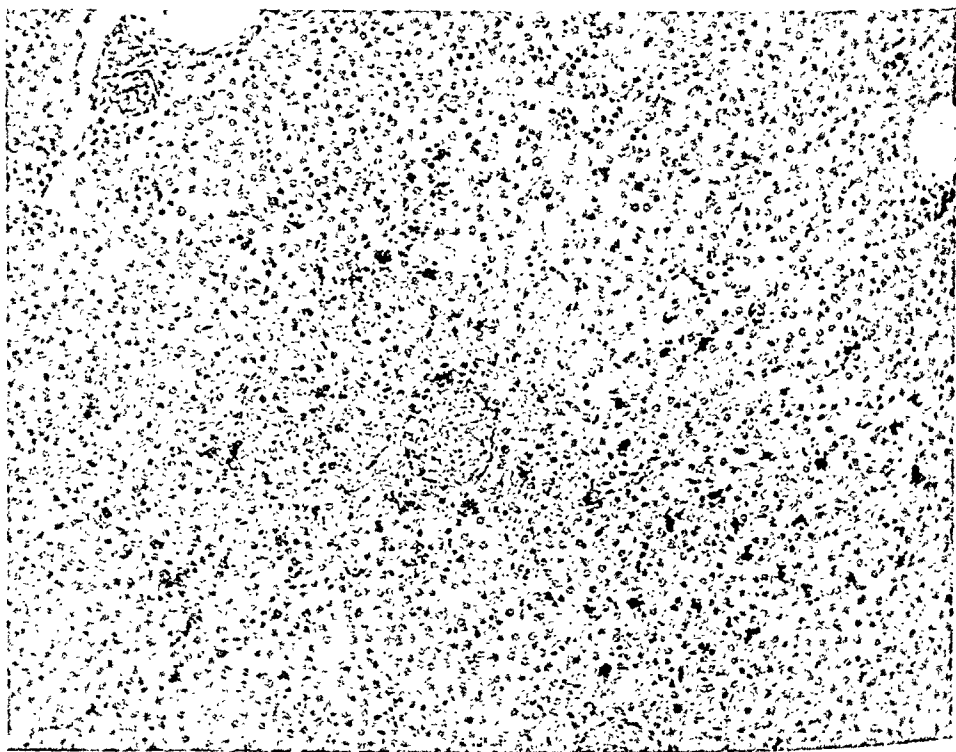


Fig. 4.—Photomicrograph of liver following fatal shock ( $\times 225$ ). There is prominent atrophy of liver cords near the central vein and veno-sinusoidal stasis.

Microscopic examination of the viscera and peritoneum following fatal shock revealed the widespread changes described by Moon.<sup>1</sup> The capillaries and venules of the lungs, kidneys, suprarenal cortex, liver (sinusoids), and omentum were diffusely dilated and stuffed with red blood cells. Pulmonary edema was marked, and the cortex of the suprarenal glands displayed prominent infiltration with polymorphonuclear neutrophilic leucocytes. The liver cords near the central vein displayed prominent atrophy, and in one liver there was fairly widespread fatty metamorphosis. Cells of the renal convoluted tubules were granular and contained frequent small vacuoles. The spleen was contracted and almost bloodless. Sections in the region of the muscle implants revealed marked acute inflammation of peritoneum and surrounding structures,



focal proteolysis of the implanted muscle, marked infiltration of neutrophilic leucocytes with focal degeneration (karyorrhexis) of the leucocytes. The leucocytic infiltration extended into the bowel wall and the addominal muscles.

Examination of the peritoneum of the surviving animals weeks later displayed encapsulation of the muscle remnants by omentum with a partially fibrous wall. The exudate changed and became predominantly composed of numerous macrophages (chronic inflammation).

In one animal dying in shock the peritoneal cavity contained 500 c.c. of a watery fluid. The other animals revealed a scanty amount of bloody peritoneal fluid. No other serious effusions were observed.

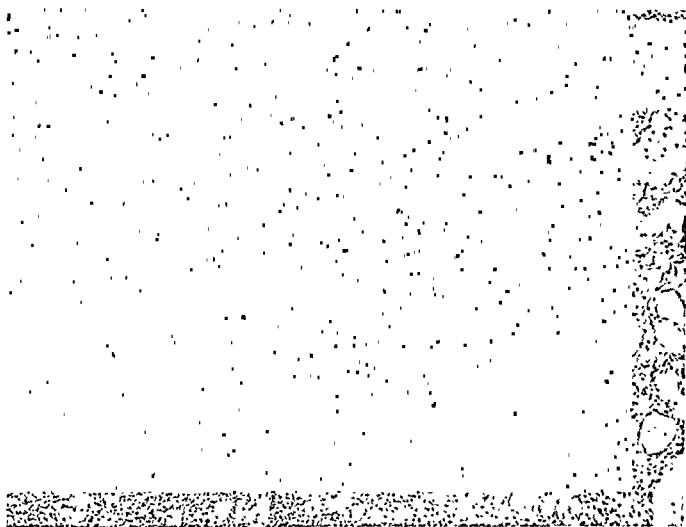


Fig. 5.—Photomicrograph of peritoneal region in fatal case ( $\times 150$ ). There is prominent edema of adipose tissue with fibrin deposition in enlarged interstices. In addition, the outstanding infiltration by polynuclear neutrophilic leucocytes is demonstrated about the implanted muscle fragments.

#### COMMENT

The intraperitoneal implantation of previously desiccated skeletal muscle is capable of producing fatal shock in dogs in the same manner as Moon<sup>1</sup> has demonstrated undesiccated muscle to produce fatal shock. The dose necessary for consistent fatal shock apparently exceeds 5 grams of dry muscle substance per kilogram body weight, since with doses of this size and less, only 3 out of 7 dogs died in shock. Since Moon<sup>1</sup> has observed consistent fatal shock with such doses of unchanged muscle substance, possibly the desiccation reduces the effectiveness of the shock-producing agent.

The local lesion at the site of the implants was inflammatory in nature. At first the inflammation was acute with prominent edema of the peritoneum and surrounding tissues, lymphatic dilatation, and infiltration by polymorphonuclear neutrophilic leucocytes. Later there was fibrous proliferation about the implants with partial absorption of the muscle by macrophages. In no section did we encounter microscopic evidence of bacterial contamination.

Most of the features in this type of shock resembled those of freezing shock.<sup>7</sup> The degree of venous hemoconcentration was quite comparable, and the findings in the viscera were similar, except for the peritoneal inflammation associated with the muscle implantation.

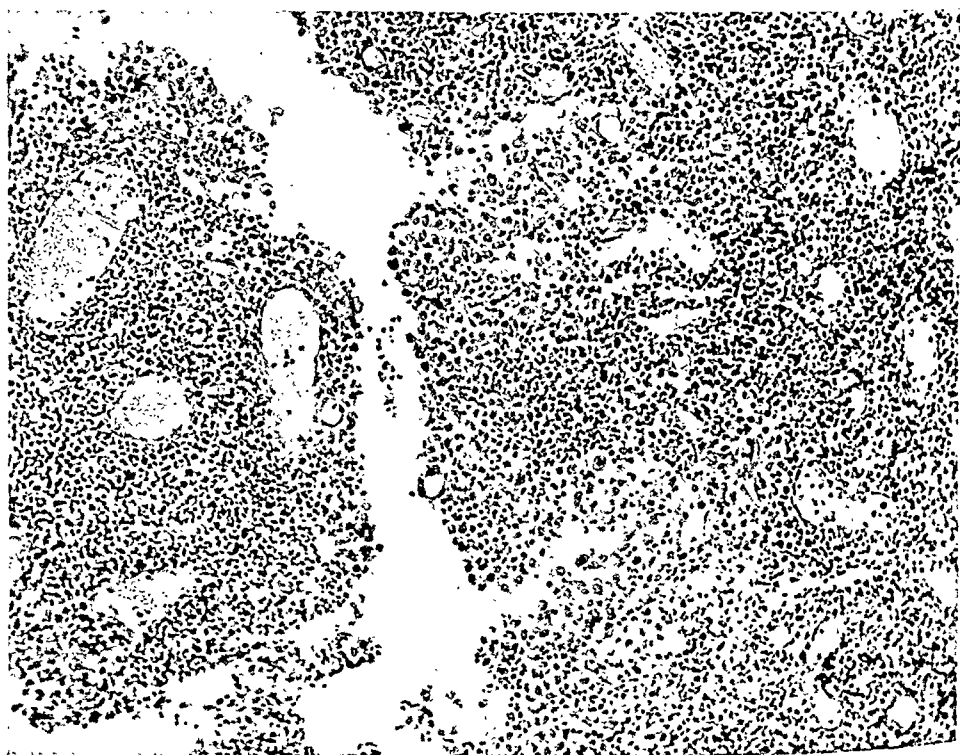


Fig. 6.—Photomicrograph of peritoneal region after recovery (X225). Macrophages are prominent in addition to remaining leucocytes.

A greater portion of the bulk of the muscle fragments remained in the peritoneal cavity after recovery. Apparently once the hemoconcentrating effects (circulating plasma loss) were overcome, the muscle substance lost its shock-producing qualities and persisted as a foreign body in the peritoneal cavity.

The decrease in plasma protein concentration (plasma sp. gr.) was continuous with the muscle implantation group and culminated in a state of hypoproteinemia. Such decrease in plasma protein concentration must represent the passage of a substantial amount of fluid into the plasma stream and has not been observed during freezing shock.

Two features in the microscopic study were of interest. First, there was the marked central atrophy of the liver encountered (see photomicrograph). The liver cord cells near the central vein were thin and narrow, giving the sinusoids a widened appearance. This feature and fatty degeneration have been observed in certain experiments on freezing shock.<sup>7</sup> Second, there was the lack of marked capillary dilatation and stasis in random samples from the general somatic musculature. This finding would seem to indicate that the capillaries of the viscera and peritoneal surfaces are the ones most prominently involved. The same observation has been made in the state of shock following a severe freeze of one hind extremity of a dog.

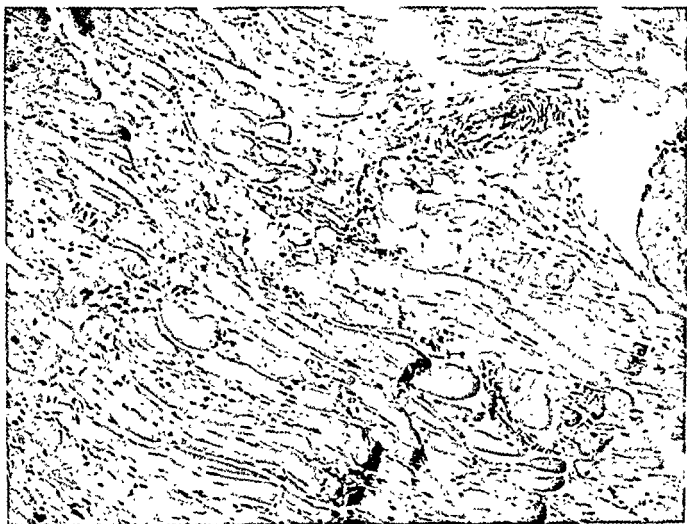


Fig. 7.—Photomicrograph of skeletal muscle following fatal shock, from region remote from peritoneum ( $\times 225$ ). Most of the capillaries are closed.

The fact that in the dogs surviving, the muscle pulp after a period of time, measurable in hours (up to 22 hours in the present group), loses its ability to promote oligemia and hemoconcentration and by all indications becomes an inert foreign substance supports strongly the contention of Moon<sup>1</sup> that substances capable of producing capillary damage are absorbed from the muscle pulp. The absorption of such substances must be the predominant cause of the oligemia. In another form of experimental shock, resulting from freezing and thawing of a hind limb of a dog,<sup>7</sup> the loss of plasma fluid into the damaged limb seems to be the predominant cause of the oligemia. Both types of shock give rise to a similar appearance of the viscera.

## CONCLUSIONS

Reconstituted desiccated muscle is capable of producing fatal shock in dogs when it is implanted in the peritoneal cavity. For consistent fatal shock a dose greater than 5 grams per kilogram seems necessary.

The changes in the viscera are identical to those following freezing shock. A dilution of the circulating plasma proteins was noted where following a freeze no change or concentration of the plasma proteins had been observed.

The fact that after a period of time in the survival group the muscle substance loses its shock-producing qualities, i.e., its ability to lower the plasma volume and cause hemoconcentration, is evidence in favor of the absorption of shock-producing substances. This feature is in contradistinction to shock following a severe freeze of one hind extremity where local plasma fluid loss appears to be the predominant etiologic factor.

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## ACUTE MACROCYTIC HEMOLYTIC ANEMIA OCCURRING FOLLOWING ADMINISTRATION OF SULFADIAZINE\*

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WE HAVE recently had opportunity to observe the development of an acute macrocytic hemolytic anemia in a patient with bronchial asthma who was receiving sulfadiazine for the treatment of bronchopneumonia. A high titer of the cold agglutinin was present also in the plasma of this patient. There have been a number of reports of hemolytic anemia following administration of sulfanilamide, but it has been observed less frequently with the use of sulfadiazine.<sup>1, 2, 5</sup> We feel, therefore, that this case is of sufficient interest to report.

### CASE REPORT

Mrs. K. K., aged 39 years, had suffered from hay fever and bronchial asthma since the age of 16. There had been progression in the severity of her asthmatic exacerbations, and hospitalization was necessary in October, 1942, for an especially severe attack. The patient was re-admitted to the hospital on Jan. 24, 1943. She had had paroxysmal dyspnea, cough, and fever for a week prior to admission; vomiting and headache had been present for 24 hours.

Examination at the time of admission to the hospital showed an obese white female weighing 98.6 kilograms, severely dyspneic, moderately cyanotic, and complaining of severe headache. Her temperature was 104.4° F. (rectal), pulse 110 per minute, respirations 24 per minute. There were asthmatic râles and wheezes throughout both lung fields. The râles were coarser and more abundant over the left base posteriorly where the percussion note and transmission of breath sounds were impaired. The blood pressure was 110 millimeters of mercury systolic and 60 millimeters diastolic. The physical findings in the remainder of the examination were within normal limits. Repeated examinations of the sputum showed a predominance of streptococci; no acid-fast organisms were present. The Wassermann test of the blood was negative. The other pertinent laboratory findings are shown in Table I.

The clinical impression at this time was that the patient had a bronchopneumonia in the left lower lobe superimposed upon a chronic bronchial asthma.

With symptomatic and general supportive treatment, progress was satisfactory for the first forty-eight hours and the temperature descended to 100.6° F. (rectal). On the evening of January 26 the patient experienced a sudden chill and elevation of temperature to 103.2° F. (rectal). Sulfadiazine was then started and continued in the dosage shown in the table. Roentgenoscopic examination of the chest on January 28 showed a number of densities about one centimeter in diameter throughout the left lung field. The right lung appeared to be clear at this time. The sulfadiazine was discontinued on January 31, as there appeared to be no clinical improvement from its use. On this day urinalysis showed the presence of many crystals of sulfadiazine; the specific gravity of this specimen of urine was 1.017, albumin and casts were absent, and the total urine output for that 24-hour period was 1400 c.c. In the next twenty-four hours a marked elevation of temperature occurred together with clinical signs of extension of the pneumonia to the right lung, and this was confirmed by roentgenoscopic examination. The sulfadiazine was resumed for a period of twenty-one hours, in which time 8 Gm. were given. It was discontinued the evening of February 3 because of the alarming clinical appearance of the patient. Marked pallor and extreme prostration were present.

\*From the Department of Medicine, Great Falls Clinic.  
Received for publication, Sept. 3, 1943.

TABLE I

CLINICAL AND LABORATORY DATA OF PATIENT WHO DEVELOPED ACUTE MACROCYTIC HEMOLYTIC ANEMIA FOLLOWING ADMINISTRATION OF SULFADIAZINE

DATE	DAYS OF ILLNESS	HIGHEST DAILY TEMPERA- TURE (RECTAL) F.	SULFADI- AZINE TOTAL 24- HOUR DOSAGE GRAMS	HEMO- GLOBIN GRAMS PER 100 C.C.	ERYTHRO- CYTE COUNT IN MILLIONS	LEUCOCYTE COUNT	NEUTRO- PHILE PER- CENTAGE	RETICULO- CYTE PER- CENTAGE	MEAN COR- PUSCULAR VOLUME IN CUHC MICRONS	BLOOD UREA MG. PER 100 C.C.	BLOOD BILIRUBIN MG. PER 100 C.C.
Jan. 24	8	104.4		13.5	5.38	12,200	81				
Jan. 25	9	103.2				10,650	81				
Jan. 26	10	103.2									
Jan. 27	11	102.6	4.0								
Jan. 28	12	101.8	6.0	12.0	4.62	11,250	85				
Jan. 29	13	101.6	6.0								
Jan. 30	14	102.4	6.0			22,200	85				
Jan. 31	15	103.4	3.0								
Feb. 1	16	103.6									
Feb. 2	17	102.2	2.0								
Feb. 3	18	100.0	6.0								
Feb. 4	19	101.4		7.5	2.90	38,000	82		124.0	65	2.9
Feb. 5	20	101.6		7.0	2.20	41,000	90				
Feb. 6	21	101.2		6.5	1.49	49,350	89	15.8			
Feb. 7	22	99.0									
Feb. 8	23	99.2		3.75	1.27	20,500	92	11.5		63	
Feb. 9	24	99.2		3.75	1.47			18.5			
Feb. 10	25	99.2		5.5	1.75	17,050	85	23.6		52	
Feb. 11	26	99.6		7.0	2.22				118.0		
Feb. 13	28	99.2		7.0	1.86						
Feb. 15	30	99.6		9.1	2.65	14,000	75	18.1			0.6
Feb. 17	32	99.6		10.0	3.04	11,400	70	11.5	125.0	28	
Feb. 18	33	99.6		10.3	3.70	7,900	56	23.3		28	
Feb. 20	35	99.6		10.3	3.70	13,500	79	15.0	97.2	20	
Feb. 21	36	99.4		10.5	3.70	7,300	69	12.0			
Feb. 26	41	99.4		10.2	4.23	6,850	51	6.3	81.3		
March 2	45	99.6		12.0	4.24		48	3.3			
March 3	46	99.2		12.0	4.27	7,850		0.5	101.8		
March 5	48	100.0		12.2	4.89			0.5	93.9		
March 31		98.4		15.7	5.72	6,800	69				
						10,550	62				

On the following morning the laboratory evidence of the blood changes shown in the table was obtained. Forty-two hours after the last dose of sulfadiazine was administered on February 3, the level of the drug in the blood was still 2.7 mg. per 100 c.c. Granular casts were present in the urine from February 6 to February 9. The urine was examined for hemoglobinuria on several occasions but none was present.

Examination of the stained smears of the peripheral blood on February 4 at the time that the hemolytic anemia was first apparent showed anisocytosis, poikilocytosis, and a considerable number of nucleated red blood cells. The leucocytes were normal except for a marked shift to the left with an increased number of band forms. Considerable difficulty was experienced in preparing blood smears as a result of the clumping of the erythrocytes. Because of this difficulty and because all individuals whose blood was of the same group as the patient's failed to cross match, the presence of the cold agglutinin was suspected and this was found to be present. The reaction of the autoagglutination which was present in the patient's plasma was accelerated by cold and was reversible at 37° C. These changes persisted in the blood during the next four days, during which time the hemoglobin decreased further to 3.75 Gm. per 100 c.c. and the erythrocyte count to 1.27 millions. There was a marked reticulocytosis, and the cold agglutinin remained actively present. The patient was slightly icteric during this period and the blood bilirubin was 2.9 mg. per 100 c.c. The qualitative van den Bergh reaction was delayed. The patient was much too ill during this time to permit collection of feces for quantitative determination of urobilinogen. There were 2.4 and 3.0 mg. of urobilinogen per day in the urine (obtained by indwelling catheter) on February 6 and February 8 respectively. The fragility test of the blood was normal. The platelet count was 249,000. Bleeding and clotting times were normal. Aside from the rise in blood urea (see table), the other metabolites of the blood including cholesterol, chlorides, carbon dioxide combining power, and the plasma proteins were normal.

During the period from February 3 to 7, the patient continued to fail clinically. She was comatose, incontinent, and there was elevation of temperature and pulse rate. She remained at a semioriented level until February 10, when she began to show signs of clinical improvement, which continued slowly but steadily until discharge from the hospital. Supportive measures included: oxygen administered constantly from January 31 to February 10 and intermittently to February 17; adrenalin, minims 5 every hour from January 26 to February 11 and intermittently thereafter; ephedrine and occasional doses of aminophylline; and intravenous supplements of 5 per cent glucose in distilled water whenever a diminishing urinary output indicated their need. During this entire period, however, urinary output was never allowed to decrease below 950 c.c. in a 24-hour period and in most instances exceeded 1200 c.c. On purely empirical grounds, liver extract was administered intramuscularly in a dosage of 90 units on February 9 and February 10, and it was continued in a dosage of 15 units daily thereafter for two weeks because of the coincidental improvement following the massive doses. The clinical response preceded any favorable changes in the blood.

Examination of the fundi appeared to give a good indication of the destructive effect on the vascular bed coincident to the hemolytic anemia. As the patient's condition gradually improved, she became aware of the fact that she had suffered a loss of visual acuity. Examination of the fundi on February 19 by Dr. A. L. Weisgerber showed the presence of many retinal hemorrhages. The deeper ones were round, sharply circumscribed, and of a dark red color. One of these had a sharply punched out center. Superficial hemorrhages were also present but were not as abundant as the deeper ones. The discs were normal. The blood vessels were of normal size and mildly tortuous. The loss of vision was accounted for by the presence of many hemorrhages in the macular areas. No fresh hemorrhages appeared after this time, and by the time the patient was discharged from the hospital on March 6 there was considerable improvement in the appearance of the fundi. By that time several of the hemorrhages had been absorbed and the remaining ones were gradually fading. Visual acuity had improved to the point that the patient was able to read large news print.

At the time the patient was discharged from the hospital on March 6, she was still weak, although she had been allowed to be up in a chair for short intervals. The asthma was still present and required an occasional injection of adrenalin. The cold agglutinin could no longer be demonstrated in the blood, having disappeared a week previously. The reticulocytes had

returned to normal, and examination of the morphology of the peripheral blood showed no significant change from the normal.

Since discharge from the hospital, the patient has been seen at the Clinic on two occasions. On March 31, hemoglobin had increased to 15.7 Gm. per 100 c.c., and the erythrocyte count to 5,720,000. When last seen on May 14, she had been well except for an occasional attack of asthma.

#### DISCUSSION

Peterson, Ham, and Finland<sup>2</sup> have recently reported three cases in which acute hemolytic anemia occurred in patients suffering from primary atypical pneumonia of unknown etiology. Each of these patients had received sulfathiazole or sulfadiazine. In two of these patients a reversible autohemagglutinin (cold agglutinin) was present. The authors found in addition that the cold agglutinin was present in the serum of the great majority of patients with primary atypical pneumonia; a large percentage of those patients showing increased concentrations of autoagglutinins had not received sulfonamide therapy during the course of their illness. Reisner and Kalkstein<sup>6</sup> in an extensive review of the literature up to 1942 were able to collect only 54 cases of autoagglutination. The recent reports by Peterson, Ham, and Finland<sup>2</sup> and of Horstmann and Tatlock,<sup>3</sup> however, suggest that this condition occurs with greater frequency than has been previously recognized.

Watson and Spink<sup>7</sup> observed an increased hemoglobin metabolism following the administration of sulfanilamide and sulfapyridine, associated with a tendency to the development of a macrocytic, hypochromic type of anemia. However, the actual factors which appear to accelerate the development of anemia in certain sensitive (?) individuals remain unknown.

The many retinal hemorrhages and the loss of visual acuity which occurred in this patient warrant further mention. Peterson, Ham, and Finland<sup>2</sup> observed that phlebothromboses and pulmonary emboli occurred in certain of their patients showing cold agglutinins in the blood. It does not appear improbable therefore that the retinal hemorrhages seen in this patient were associated with intravascular clumping of the erythrocytes, thrombus formation, and subsequent damage to the wall of the blood vessels, leading to hemorrhages outside the vessel wall.

Improvement in the patient's condition occurred coincident to the administration of 90 units of liver extract (15 units per cubic centimeter) on two successive days. At this time there was no evidence that this therapy was specific in its action, and that recovery might not have ensued without its use. The recent report of Kornberg, Daft, and Sebrell,<sup>4</sup> however, does offer some experimental evidence to suggest that the liver extract may have had a role in the recovery of this patient. These authors observed that rats fed sulfathiazole, sulfadiazine, or sulfanilamide at a one per cent level in purified diets developed a severe granulocytopenia or anemia, or both. Treatment with certain liver fractions administered orally succeeded in correcting the granulocytopenia and the anemia in spite of continued ingestion by these animals of the sulfonamide-containing diet.

#### SUMMARY

1. A case of acute macrocytic hemolytic anemia which occurred following sulfadiazine therapy for an atypical pneumonia is reported.



2. A true reversible cold hemagglutinin was present in the plasma of this patient during the period of acute illness. The possible relation of this phenomena to the changes occurring in the retinae and the resulting loss of visual acuity is discussed.

3. Recovery of the patient occurred coincident to the administration of liver extract. Recent experimental observations in animals would appear to justify further study of the effect of this therapy in the prevention and treatment of macrocytic hemolytic anemia in the human being, particularly those cases occurring subsequent to the administration of the sulfonamides.

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# MICROSCOPIC OBSERVATION OF COLLODION PARTICLE AGGLUTINATION IN PROTEIN-ANTIPROTEIN SYSTEMS\*

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IT HAS been shown that it is possible to obtain antigen-antibody reactions on the surface of collodion particles, thus converting precipitin or flocculating type of reactions into agglutinations.<sup>1-7</sup> A comparison of the collodion particle agglutination technique with other methods of determining antigen and antibody titers has also been reported.<sup>8-11</sup>

The object of this report is to show that, by means of a microscopic technique, antibodies can be detected at titers much higher than those obtained by the precipitation test or by the macroscopic collodion particle agglutination method.

## EXPERIMENTAL

Collodion particles were prepared as described in a previous communication.<sup>9</sup> Three comparative sets of experiments were carried out simultaneously for each protein and its homologous rabbit antiprotein antibody, namely: (a) an ordinary precipitation test, (b) a macroscopic collodion particle agglutination test, and (c) a microscopic collodion particle agglutination test.

The precipitation tests were made by adding 0.5 c.c. of antigen (diluted 1:10 in saline) to 0.5 c.c. of antiserum dilutions. The mixture was shaken thoroughly, placed in the refrigerator overnight, and read the following day.

The macroscopic collodion particle agglutination tests were made by first sensitizing the collodion particles with antigen. This was accomplished by centrifugalizing, for five minutes at 3000 revolutions per minute, 5 c.c. of the stock collodion particle suspension. The aqueous supernate was discarded and the sediment resuspended in 10 c.c. of antigen (diluted 1:10 in saline). This suspension was placed in the refrigerator overnight and centrifugalized for one to two minutes at 3000 revolutions per minute the following day. All but 0.5 c.c. of the supernate was decanted; then 9.5 c.c. of saline were added to the sediment and mixed thoroughly to give a homogeneous suspension. The particles so sensitized constituted the antigen. For the test, 0.2 c.c. of each antiserum dilution was added to 0.2 c.c. of sensitized particles (antigen) in agglutination tubes. The tubes after thorough shaking were placed in the refrigerator overnight, and on the following day 0.5 c.c. of saline was added to each tube. The tubes were then centrifugalized in the angle centrifuge for one to two minutes at 1500 revolutions per minute, and the agglutination was observed by gently tapping the bottom of the tube a few times, thereby resuspending the sediment. The addition of the 0.5 c.c. of saline, as stated above, and the use of a mag-

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nifier glass greatly facilitated the readings. A microscopic examination of each suspension showed a twofold or fourfold increase in the titer, but in most instances a clear-cut final titer or end point was not obtained because the antigen controls in some experiments or the antiserum controls in other experiments contained clumps which were not detectable macroscopically but resulted in anomalous readings in the region of the end point when examined microscopically

TABLE I  
CHICKEN-ANTICHICKEN SYSTEM

TUBE	ANTI-SERUM DILUTION 1:	READING	REMARKS
<i>Ordinary Precipitation Test</i>			
1	5	+	Antigen control: 0.5 c.c. chicken serum (1:10) + 0.5 c.c. saline.
2	10	±	
3	20	Sl. cloud	
4	40	-	Antigen + pneumococcus antiserum (rabbit) control: 0.5 c.c. chicken serum (1:10) + 0.5 c.c. of an antipneumococcus serum (1:5)
5	80	-	
6	160	-	
Antigen control		-	
Antigen + pneumococcus antiserum (rabbit) control		-	
<i>Macroscopic Colloidon Particle Agglutination Test</i>			
1	5	+	Antigen control: 0.2 c.c. chicken serum sensitized colloidon particles + 0.2 c.c. saline.
2	10	+	
3	20	±	
4	40	-	Antigen + pneumococcus antiserum control: 0.2 c.c. sensitized particles + 0.2 c.c. of an antipneumococcus serum.
5	80	-	
6	160	-	
7	320	-	
8	640	-	
9	1280	-	
10	2560	-	
Antigen control		-	
Antigen + pneumococcus antiserum control		-	
<i>Microscopic Colloidon Particle Agglutination Test</i>			
1	5	4+*	Antigen control: 0.02 c.c. colloidon particles + 0.1 c.c. antigen (1:10) + 0.1 c.c. saline.
2	10	3+*	
3	20	2+±	
4	40	2+	Antigen + pneumococcus antiserum control: 0.02 c.c. c.p. + 0.1 c.c. antigen (1:10) + 0.1 c.c. of an antipneumococcus rabbit serum.
5	80	2+	
6	160	±±	
7	320	+	
8	640	+	Colloidon particle control: 0.02 c.c. c.p. + 0.2 c.c. saline.
9	1280	± (-)	
10	2560	± (-)	
11	5120	± (-)	Antiserum controls: 0.02 c.c. c.p. + 0.1 c.c. antiserum dilution + 0.1 c.c. saline.
12	10240	± (-)	
Antigen control		±†	
Antigen + pneumococcus antiserum control		-	
Colloidon particle control		-	

\*Visible precipitate containing enmeshed colloidon particles.

†The antigen control showed many small clumps and single particles in a ratio of about 1:1 and is recorded as ±. Therefore, tubes 9, 10, 11, and 12 which also showed ± reactions are recorded as (-) negative.

The microscopic tests, which form the basis of this report, were carried out essentially in a manner described previously.<sup>9</sup> In the present tests 0.02 c.c. of colloidon particle stock suspension was transferred to agglutination

tubes. Then 0.1 c.c. of antigen (diluted 1:10 in saline) was added and mixed thoroughly. The tubes were allowed to stand for fifteen minutes, and finally 0.1 c.c. of antiserum dilution was added. The tubes were rotated vigorously to assure thorough mixing and placed in the refrigerator overnight. The next day, a drop of the mixture was placed on a plain glass slide. Next to it was placed a drop of the antigen control mixture, and finally a third drop containing the antiserum control mixture was placed on the slide. Each drop was covered with a cover glass and examined under the oil immersion lens. An explanation of the controls appears in the tables following.

TABLE II  
EGG WHITE-ANTI EGG WHITE SYSTEM

TUBE	ANTI-SERUM DILUTION 1:	READING	REMARKS
<i>Ordinary Precipitation Test</i>			
1	2.5	+	Antigen control: 0.5 c.c. egg white (1:10) + 0.5 c.c. saline.
2	5	±	
3	10	Cloudy	
4	20	-	Antigen + pneumococcus antiserum control: 0.5 c.c. egg white (1:10) + 0.5 c.c. of an antipneumococcus rabbit serum (1:5).
5	40	-	
6	80	-	
Antigen control	-	-	
Antigen + pneumococcus antiserum control	-	-	
<i>Macroscopic Collodion Particle Agglutination Test</i>			
1	5	+	Antigen control: 0.2 c.c. egg white sensitized collodion particles + 0.2 c.c. saline
2	10	+	
3	20	+	
4	40	±	Antigen + pneumococcus antiserum control: 0.2 c.c. sensitized particles + 0.2 c.c. of an antipneumococcus rabbit serum.
5	80	-	
6	160	-	
7	320	-	
8	640	-	
9	1000	-	
10	3200	-	
Antigen control	-	-	
Antigen + pneumococcus antiserum control	-	-	
<i>Microscopic Technique</i>			
1	5	4+*	Antigen control: 0.02 c.c. collodion particles + 0.1 c.c. antigen (1:10) + 0.1 c.c. saline.
2	10	3+*	
3	20	2+	
4	40	±	Antigen + pneumococcus antiserum control: 0.2 c.c. c.p. + 0.1 c.c. antigen (1:10) + 0.1 c.c. of an antipneumococcus rabbit serum.
5	80	+	
6	160	+	
7	320	-	Collodion particle control: 0.02 c.c. c.p. + 0.2 c.c. saline. Was negative.
8	640	-	
9	1280	-	
10	2560	-	Antiserum controls: 0.02 c.c. c.p. + 0.1 c.c. antiserum dilution + 0.1 c.c. saline. Were negative.
Antigen control	-	-	
Antigen + pneumococcus antiserum control	-	-	

\*Visible precipitate with enmeshed collodion particles.

#### RESULTS

Seven protein-antiprotein systems were studied; namely, horse, hog, human, bovine, sheep, chicken, and egg white. The antisera to these proteins were prepared in rabbits by the intra-abdominal injections of the antigens.

TABLE III  
BOVINE-ANTIBOVINE SYSTEM

TUBE	ANTI-SERUM DILUTION 1:	READING	REMARKS
<i>Ordinary Precipitation Test</i>			
1	5	+	Controls were prepared similar to those in Tables I and II except that bovine and antbovine sera were used.
2	10	+	
3	20	±	
4	40	-	
5	80	-	
6	160	-	
Antigen control		-	
Antigen + pneumococcus rabbit antiserum control		-	
<i>Macroscopic Colloidon Particle Agglutination Test</i>			
1	5	+	Controls were prepared similar to those in Tables I and II except that bovine and antbovine sera were used.
2	10	+	
3	20	+	
4	40	+	
5	80	±	
6	160	-	
7	320	-	
8	640	-	
9	1280	-	
10	2560	-	
Antigen control		-	
Antigen + pneumococcus antiserum control		-	
<i>Microscopic Technique</i>			
1	20	3+*	Controls were prepared similar to those in Tables I and II except that bovine and antbovine sera were used.
2	40	2+	
3	80	±	
4	160	±	
5	320	+	
6	640	± (-)	
7	1280	± (-)	
8	2560	± (-)	
9	5120	± (-)	
10	10240	± (-)	
Antigen control		±	
Antigen + pneumococcus antiserum control		±	
Colloidon particle control		-	
Antiserum dilutions control		±	

\*Visible precipitate with enmeshed colloidon particles.

TABLE IV  
SUMMARY OF SOME OF THE PROTEIN-ANTIPROTEIN REACTIONS

SYSTEM	HIGHEST TITER BY ORDINARY PRECIPITATION TEST. ANTI-SERUM DILUTION METHOD	HIGHEST TITER BY MICROSCOPIC COLLODION PARTICLE AGGLUTINATION TEST	HIGHEST TITER BY MICROSCOPIC COLLODION PARTICLE AGGLUTINATION TEST	HIGHEST TITER BY ANTIGEN DILUTION METHOD—ORDINARY PRECIPITATION TEST
Egg white-antegg white	1:10	1: 40	1: 320	1:50,000
Sheep-antisheep	1:10	1:100	1:1000	1:50,000
Hog-antihog	1:10	1: 80	1: 160	1:40,000
Bovine-antibovine	1:20	1: 80	1: 320	1:80,000
Horse-antihorse	1:10	1: 40	1: 160	1:80,000
Chicken-antichicken	1:10	1: 20	1: 640	1:40,000
Human-antihuman	1: 5	1: 50	1: 100	1:20,000

Tables I, II, and III give the results of comparative tests using the three methods described above; for the chicken-antichicken, egg white-antiegg white, and bovine-antibovine systems. Table IV is a summary of the results obtained with some of the protein-antiprotein systems studied and includes the highest titer obtained by the antigen dilution method in the ordinary precipitation test.

#### SUMMARY AND CONCLUSIONS

A microscopic method using a collodion particle agglutination technique for detecting the presence of specific antibody in several antiprotein sera is described. In higher dilutions of the various antisera studied, the method gives positive results while negative results are obtained at the same titer by either the ordinary precipitation test or the macroscopic collodion particle agglutination test.

I wish to thank Dr. Jules Freund for his suggestions and criticisms throughout this study.

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# BILATERAL ADRENAL HEMORRHAGE (WATERHOUSE-FRIDERICHSEN SYNDROME) ASSOCIATED WITH MENINGOCOCCAL SEPTICEMIA\*

REPORT OF FOUR CASES IN ADULTS WITH A REVIEW OF THE LITERATURE

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IN a series of 182 cases of meningococcal septicemia seen at Station Hospital during the winter of 1942-43, special interest centers on the four cases of massive bilateral adrenal hemorrhage with purpura, recognized as Waterhouse-Friderichsen syndrome.

No attempt will be made in this paper to review the literature on this subject previous to 1939, as the excellent reviews of Aegerter,<sup>1</sup> Sacks,<sup>2</sup> and Kunstadter<sup>3</sup> bring the total published cases to 73. Lindsay, Rice, Selinger, and Robbins<sup>4</sup> collected 14 more cases and added seven of their own, making a total of 94 reported cases up to 1941. Since that article, Grace, Harrison, and Davie<sup>5</sup> added three cases; Moss and Schenken,<sup>6</sup> Drummond and Tooke,<sup>7</sup> and Taylor and Kean<sup>8</sup> each added two cases; and Hughes,<sup>9</sup> Mallory,<sup>10</sup> Fox,<sup>11</sup> Leone,<sup>12</sup> Kwedar,<sup>13</sup> Monfort and Mehrling,<sup>14</sup> Michael and Jacobus,<sup>15</sup> and Gollick<sup>16</sup> added one case each, bringing the total of reported cases to 111. In 1942, Banks and McCartney<sup>17</sup> reported ten cases of meningococcal encephalitis. In reviewing this article we find that two of their cases appear to involve the adrenals sufficiently to be classified as Waterhouse-Friderichsen syndrome. The addition of these, together with our four cases, brings the number of published cases to date to 117.

We have reviewed the articles by Barsoum<sup>18</sup> in 1936 and Thorstad<sup>19</sup> in 1942. Their cases were of adrenal apoplexy, but cannot, we believe, be classed as Waterhouse-Friderichsen syndromes. In Thorstad's review of the literature, he collected 22 cases of adrenal apoplexy. Only three of these were due to

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syndrome was first accurately described in the literature Cases of Purpura Ending Fatally Associated with Hemorrhage, Brit. J. Dermat. 13: 445-467, 1901 (Cases 1 to 12). The onset, malaise, restlessness, and often gastrointestinal disturbance by lethargy, which rapidly deepens into coma. High fever, weak rapid pulse, intense cyanosis, acteristic. The disease is usually fatal in lateral adrenal hemorrhage is the most in from 60 to 70 per cent of the cases, cases, pneumococcus, hemolytic streptococci have been isolated. he skin are char- bers. Massive, bi- The etiology is, In the remaining illus, and *B. coli*

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The report on bacteriologic studies in this series has been written by the above authors and published under the title "Purpuric Lesions in Meningococcal Infections" (V. LAM. & CLIN. MED. 29: 273, 1944). The clinical report is being written by Captains C. M. Dummer and J. L. Cloninger and will be published at an early date.

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In reviewing Kunstadter's article, we find the total published cases at that time to be 73 instead of the 74 mentioned by Lindsay, Rice, Selinger, and Robbins. Thus, the addition of 21 cases by these authors brings the total to 94 instead of the 96 mentioned by them.

infection, and we have been unable to review these cases. We have been unable to review the two articles by Landis<sup>29</sup> on the Waterhouse-Friderichsen syndrome.

Inasmuch as about 86 per cent of all cases reported are in children under nine years, the four cases to be presented are of some interest as the age ranges from 18 to 21 years. Aegerter's<sup>1</sup> review of the literature found only six cases of Waterhouse-Friderichsen syndrome occurring in adults, and Foucar,<sup>20</sup> in 1936, reported one case in a twenty-year-old male. Kvedar<sup>13</sup> reports one case of fulminating massive hemorrhage into both adrenals with purpura in a 58-year-old white female. Leone<sup>12</sup> reports a case in an adult alcoholic with negative bacteriologic findings. In addition, the above-mentioned cases of Hughes<sup>9</sup> and Drummond and Tooke<sup>7</sup> and one case each of Grace, Harrison, and Davie<sup>9</sup> and Banks and McCartney<sup>17</sup> occurred in adults. Including the four cases presented here, there are a total of eighteen cases occurring in adults reported in the literature to date.

#### CHART 1

##### SUMMARY OF AUTOPSIES AND MORTALITY RATE

Case 1.	Cerebrospinal meningitis (epidemic). <i>Waterhouse-Friderichsen syndrome</i> .
Case 2.	<i>Waterhouse-Friderichsen syndrome</i> —( <i>N. intracellularis</i> ).
Case 3.	<i>Waterhouse-Friderichsen syndrome</i> —(Organism not isolated).
Case 4.	Cerebrospinal meningitis (epidemic)—Gastrointestinal hemorrhage. Confluent bronchopneumonia. Agonal neurogenic gastric ulcer.
Case 5.	Cerebrospinal meningitis (epidemic). <i>Tubular degeneration due to sulfadiazine</i> .
Case 6.	<i>Waterhouse-Friderichsen syndrome</i> —(Organism not isolated).
Number of proved meningococcal meningitis and meningococcemia cases	167
Number of deaths in this group	4
Mortality rate	2.39 per cent
Total number of cases, including 15 of undetermined etiology	182
Total number of deaths	6
Mortality rate	3.29 per cent

In Chart 1, a brief summary of the causes of death of the six cases is given. In the group of 167 proved meningococcus cases, there were four deaths or 2.39 per cent. In the total number of cases, including 15 of undetermined etiology, there were six deaths or 3.29 per cent. These 15 cases showed cloudy spinal fluids and definite clinical signs, and coming during a meningococcus epidemic it is reasonable to suppose that the meningococcus was probably the causative organism.

Cases 1 and 2 are cases of Waterhouse-Friderichsen syndrome caused by *Neisseria intracellularis* isolated either from the blood, spinal fluid, or petechiae. In Case 2, gram-negative intracellular diplococci were found on the smear from petechiae. In Cases 3 and 6, both associated with a fulminating septicemia, purpuric rash, and bilateral adrenal hemorrhage, no causative organism could be isolated. They both occurred at the height of the epidemic of cerebrospinal meningitis. Cultures and smears of the petechiae were not done at the time these cases were admitted, and in all probability this would have been a valuable aid in diagnosis.

A summary of the pathologic findings in the four cases of Waterhouse-Friderichsen syndrome showed bilateral hemorrhage into the adrenals with some variation as to the amount of hemorrhage. In one case, it was spotty in character, while in another, the hemorrhage was sufficient to convert the adrenal into



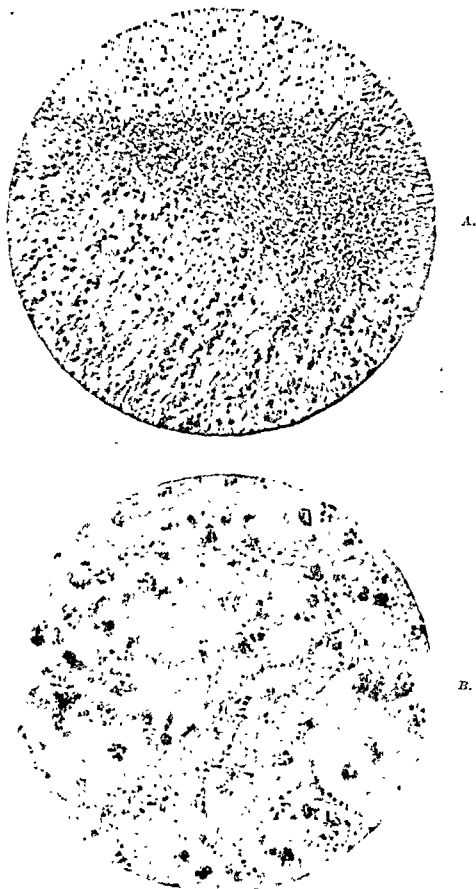


Fig 1.—*A*, low power (magnification,  $\times 130$ ) and *B*, high power (magnification,  $\times 450$ ) photomicrograph of adrenal (Case 2, Chart 1) showing extensive hemorrhage destruction of parenchyma. The fine particles in the high power are blood pigment and not bacteria. Hematoxylin and eosin stain.

a degenerated blood cyst. The weight of the glands varied between 8 and 11.5 Gm. (normal 5 to 6 Gm.). The histopathology of the adrenals usually confirms the gross findings and depends on the extent of the hemorrhage. In two cases, the surrounding periadrenal fat was hemorrhagic and the diagnosis could be made grossly before the adrenals were dissected out. In three cases, neither the gross nor microscopic examination of the brain showed any evidence of meningitis, but only congestion and edema. One case showed an obvious meningitis with only spotty adrenal hemorrhage. All cases had a petechial rash and two were cyanotic with large purpuric areas. One had petechiae on the pleura, and two showed purpuric areas on the epicardium and serosa of the small intestine. In one case both kidneys were studded with small petechiae, and in three the kidneys were intensely congested. Mesenteric lymphadenitis was noted in three, but no hyperplasia of Peyer's patches or solitary lymph nodes were found. Acute splenic tumor was found in all cases, and in one case there was persistence of the thymus. The adrenal vessels were examined for thrombi in each case but none were found. Histologic examination of petechiae from the skin and heart showed engorged vessels and some extravasation of blood into the surrounding tissue. Slight inflammatory reaction was noted in one case, but no bacteria could be found on special staining.

Case 5 recovered from the meningitis, but died from a toxic degeneration of the tubules of the kidney after prolonged sulfadiazine therapy and an associated bronchopneumonia. Histologically, the tubules, particularly the proximal and distal convoluted tubules, were swollen, and the lining cells were necrotic with no evidence of injury to the glomeruli. This case is similar to the report of Hellwig and Reed<sup>21</sup> who believe there are two types of renal lesions due to the sulfa drug therapy; namely, toxic effects on the tubular epithelium, simulating mercury poisoning, and mechanical blockage of the urinary passages. Erganian and Doval<sup>22</sup> recently published a similar case in a nine-month-old child in which they also noted areas of focal necrosis in the liver after sulfathiazole therapy. In the Case Records of the Massachusetts General Hospital published in the New England Journal of Medicine,<sup>23</sup> a similar case is found, which in addition to toxic degeneration of the tubules also showed granulomatous lesions. Maisel, McSwain, and Glenn<sup>24</sup> produced these lesions in animals, and Rake, van Dyke, and Corwin<sup>25</sup> in their experiments on animals found an occasional one with severe toxic degeneration of the tubules. Kolmer<sup>26</sup> noted similar findings in rabbits after sulfathiazole. Severe injury to the tubules, glomeruli, and vessels was noted by Luetschler and Blackman<sup>27</sup> following sulfathiazole administration in several cases.

Case 4 is of interest because of a history of vomiting with tarry stools for three days previous to admission. Twelve hours later he developed convulsions and a fulminating meningitis and died in twenty-four hours. An autopsy showed no obvious cause for the gastrointestinal hemorrhage, and it is interesting to speculate whether the internal hemorrhage was associated with or caused by the severe meningococcus infection. A purpuric skin rash was not found in this case. In view of the ectodermal tropism of the meningococcus for such structures as the skin, brain, and adrenal medulla, it might be argued that the epithelium of the gastrointestinal tract could be similarly involved. Our re-

sults on cultural studies and smears of skin lesions would indicate that they are the result of the direct action of the meningococcus on the capillaries and arterioles. The finding of gram-negative intracellular diplococci on smears from the purpuric skin lesions by McLean and Caffey,<sup>28</sup> together with our experiences, would substantiate this view. From these findings, it might be assumed that the gastrointestinal hemorrhage in this case was due to the direct involvement of the mucous membrane of the stomach and intestine by the meningococci.

#### CASE REPORTS

**CASE 1.**—W. T., aged 19 years, was admitted in coma. No history was obtainable. He was continuously restless and extremely violent at times. His pulse was 100, temperature, 99°, and there was a moderate rigidity of the neck with sparse purpura on the body and extremities. A spinal tap was finally attempted after much sedation, but the patient's condition remained critical, and he expired twenty hours after admission. Treatment consisted of saline and glucose, sulfadiazine intravenously, and blood plasma. Spinal fluid cell count was 2,900 with 95 per cent polymorphonuclears, and the smear showed gram-negative intracellular diplococci. Both the spinal fluid and blood culture were positive for *N. intracellularis*, Type I.

**Autopsy Findings.**—The neck, chest, and head contained numerous petechiae. There were a moderate number of small petechiae on the visceral pleura of the lower lobe of the left lung. There were also numerous small and large petechiae on the epicardium over the left ventricle and auricle. The spleen was enlarged and of soft consistency. The adrenal glands were enlarged, the right weighing 8.5 Gm. and measuring 6 cm.  $\times$  5 mm.; the left adrenal, weighing 8 Gm. Both showed spotty areas of congestion, and the periadrenal fat was hemorrhagic. The mesenteric lymph nodes were palpable. The superficial vessels of the brain were engorged, and there was edema causing some flattening of the convolutions. Both cerebral hemispheres were covered with a yellowish-white exudate indicative of a severe meningitis.

Microscopic examination of the cortex and medulla of both adrenal glands showed a moderate amount of hemorrhage with some degeneration of the cells. Sections from the petechiae of the pleura and the heart revealed numerous large and small hemorrhagic areas. The leptomeninges showed edema, congestion, and diffuse purulent inflammatory cell infiltration.

**CASE 2.**—J. S., aged 18 years, gave a history of onset of fever, chills, headache, vomiting, and generalized aching five hours before admission. No meningeal signs or evidence of purpura. Blood culture taken at time of admission was subsequently found to be positive for *N. intracellularis*. Spinal fluid was clear with no cells or organisms and culture was negative after 48 hours' incubation. Red blood cells, 4,620,000; hemoglobin, 90 per cent; white blood cells, 10,950 with 90 per cent segmented forms; 4 per cent staff cells; and 6 per cent lymphocytes. The next day the patient's trunk was covered with purpura and the neck was quite rigid. Blood smears from the purpuric areas showed gram-negative intracellular diplococci which on culturing proved to be *N. intracellularis*, Type I. The patient expired in 48 hours. The blood chemistry showed: Nonprotein nitrogen, 50 mg.; urea N., 20 mg.; sugar, 167 mg.; chlorides, 462 mg.; phosphorus, 3.8 mg.; calcium, 10 mg.; CO<sub>2</sub>, 45 vol. per cent; vitamin C, 1.17 mg.; prothrombin time, 87 per cent; and cell volume, 31 per cent.

**Autopsy Findings.**—Practically the entire skin surface was covered with small and large petechiae and large areas of purplish mottling. There were a few rather large petechiae on the visceral pleura of both lower lobes. The heart weighed 326 Gm., and the epicardium covering the left ventricle and auricle contained numerous petechiae. The spleen was moderately enlarged, and on sectioning was of deep red coloration. The right adrenal weighed 9 Gm. and measured 6.5 cm.  $\times$  4 cm.  $\times$  7 mm. The right adrenal was markedly congested, but there was still some evidence of pigment in some areas. The hemorrhagic areas were spotty and not confluent. The left adrenal was thickened and hemorrhagic throughout, the right one being much less involved. The surrounding fat was hemorrhagic. On exposing the brain, the

superficial vessels were congested, and there was some edema, causing a flattening of the convolutions. There was no exudate on the surface of the brain indicative of meningitis.

Histopathologic findings of the left adrenal showed massive hemorrhage into both the cortex and medulla. The cortex appeared to be more involved than the medulla and the hemorrhage was diffuse. The cells were degenerated, swollen, and contained fine particles of pigment. There were very few normal-appearing cells found in any area of the gland, and there appeared to be complete destruction of the entire left adrenal. In the right adrenal gland the hemorrhage was not so severe and appeared to be spotty rather than diffuse. The medulla and cortex were equally involved with large areas of cellular destruction in the hemorrhagic areas. Where the hemorrhage was less severe or absent, some of the cells appeared normal. The periadrenal connective tissue and fat were also hemorrhagic on both sides. Sections from the brain showed congestion and edema of the leptomeninges with no evidence of inflammatory exudate. Examination of petechiae from the skin showed engorgement of the blood vessels with some extravasation of blood into the surrounding tissue.

CASE 3.—A. G., aged 21 years, was admitted in extremis. No history was obtainable. There was a diffuse cyanosis of the entire body, more marked in fingers and toes. Through the dusky skin numerous petechiae were found. The extremities were cold; the temperature 103°. The blood pressure was unobtainable. The lungs were clear. There was no neck rigidity. Medication, oxygen, and plasma were given. The patient sank rapidly despite therapy and died two hours after admission. No ante-mortem or post-mortem laboratory examinations were done.

*Autopsy Findings.*—There was marked evidence of cyanosis of the entire body, especially the head and neck. Beneath the dusky cyanosis large and small petechiae were noted. The lungs showed terminal edema and congestion. The spleen was slightly enlarged, of firm consistency, and hemorrhagic on sectioning. The right adrenal gland measured 5.5 cm. × 5.5 cm. × 1 cm. It was triangular in shape, swollen, and hemorrhagic. The weight was 10 Gm., and on sectioning, was degenerated, hemorrhagic, and resembled a blood cyst. The left adrenal was crescentic in shape, and measured 6 cm. × 3.5 cm. × 8 mm. It weighed 8 Gm., and, likewise, was swollen and hemorrhagic throughout. The surrounding fat of both adrenals was congested. The lining mucous membrane of the small intestine was congested in a few areas, and there was no evidence of any enlarged mesenteric lymph nodes. Both kidneys were swollen and intensely hemorrhagic. The superficial vessels covering the cerebral hemispheres were engorged, and there was a moderate amount of edema of the brain, causing partial obliteration of the convolutions. There was no evidence of any exudate indicative of meningitis.

*Histopathologic Findings.*—The spleen showed congestion, and the kidneys showed scattered hemorrhagic areas in which the tubules and glomeruli were degenerated. Both adrenal glands showed extensive massive hemorrhage with almost complete destruction of all tissue elements. Both the cortex and medulla were almost completely destroyed with only a few small remnants of adrenal tissue remaining. In these latter areas the cells were swollen and appeared degenerated and vacuolated. Most of the cells contained numerous particles of brownish pigment. Special stains for meningococci were negative.

CASE 4.—A. R., aged 19 years, was admitted in shock from profuse bleeding. He gave a history of vomiting and passing of blood for three days. The blood count on admission was red blood cells, 1,900,000 with 50 per cent hemoglobin; white blood cells, 15,000 with 76 per cent polymorphonuclears, 20 per cent staff cells and 4 per cent lymphocytes. An immediate blood transfusion was given. The blood pressure was 60/50. When seen the following morning he appeared improved. One-half hour later he developed generalized convulsions and was given oxygen and transfusions. A spinal tap was done at this time; it contained 14,450 cells with 95 per cent polymorphonuclears; smears showed intracellular and extracellular gram-negative diplococci. The culture of the spinal fluid subsequently showed *N. intracellularis*, Type I and blood culture was negative. He expired twenty-four hours later.

*Autopsy Findings.*—There was no evidence of any petechial rash on the body. The lower lobe of the left lung was congested and showed evidence of consolidation. The right lung was also congested and consolidated. The spleen was enlarged, of firm consistency,

and was hemorrhagic on sectioning. The left adrenal gland appeared enlarged and contained a few small petechiae. The right adrenal was normal. The cardiac end of the stomach was perforated because of post-mortem degeneration. There was no evidence of any ulceration of the esophagus or the stomach. The mucosa showed no petechiae. The duodenum and jejunum showed no evidence of ulceration or hemorrhage. The ileum was distended with dark-colored blood which had the consistency of putty. The entire intestinal tract from the ileum to the rectum was distended with similar bloody material. On examining the mucosa of the entire intestinal tract, no evidence of ulceration or bleeding points could be found. The brain showed edema and congestion with flattening of the convolutions. The brain was covered by a thin white exudate.

*Histopathologic Findings.*—In the left adrenal, the medulla showed some congestion, but there was no evidence of diffuse congestion or degeneration in either adrenal gland. The lungs showed confluent areas of bronchopneumonia with marked evidence of terminal edema and congestion. The leptomeninges of the brain were markedly thickened, congested, and diffusely infiltrated with a purulent exudate. This purulent exudate was also found surrounding the cerebellum and brain stem. Sections taken from various parts of the stomach and intestines showed no evidence of pathology.

CASE 5.—G. H., aged 37 years, was admitted with symptoms of acute gastroenteritis. There was no headache or rigidity of the neck. The following day he became irrational, and purpura appeared on the trunk and extremities. The spinal fluid was cloudy, and *N. intracellularis*, Type I was isolated. A cell count of the spinal fluid was 24,000 with 96 per cent polymorphonuclears, and the smear showed gram-negative intracellular diplococci. The blood culture was negative. White blood cells, 29,750 with 79 per cent segmented forms, 8 per cent staff cells, 2 per cent juveniles, 11 per cent lymphocytes. Sulfadiazine therapy was instituted and the patient appeared to improve, but remained in a critical condition. On the sixth day he developed bilateral synovial effusion of the knees, and on the ninth day he appeared to be developing an acidosis. Sulfadiazine therapy was discontinued, and the nonprotein nitrogen was 120 mg. per 100 c.c. The urinary output remained adequate without albuminuria or hematuria until twenty-four hours before death, which occurred on the tenth hospital day. On the ninth day numerous red blood cells, sulfadiazine crystals, and casts appeared in the urine. A total of 63 Gm. of sulfadiazine was given during the course of the illness. There were no terminal signs of meningitis.

*Autopsy Findings.*—At the time the autopsy was performed, there was no evidence of any petechiae. The lower lobe of the right lung showed congestion and diffuse consolidation. The lower lobe on the left side was congested, but showed no evidence of pneumonia. The spleen was moderately enlarged, of soft consistency, and, on sectioning, was of a deep red color with the markings prominent. Both adrenals were of normal size and showed no evidence of degeneration or hemorrhage. The kidneys were swollen. The right kidney weighed 160 Gm. and the left kidney 165 Gm. The capsules stripped with ease and revealed a smooth congested surface. The pelves were congested and contained a moderate amount of homogeneous, brown amorphous material. A small amount of the amorphous material removed from the pelvis was tested for sulfadiazine and found to be positive. There was no reduction of the ratio between the cortex and medulla. The ureters showed no evidence of obstruction or abnormality. On examining the brain, the convolutions were flattened and the superficial vessels were congested. The leptomeninges showed no gross evidence of any meningitis.

*Histopathologic Findings.*—Numerous sections taken from both kidneys showed a widespread injury to the convoluted tubules. The epithelial cells were swollen and degenerated. The nuclei in many were stained poorly and the cytoplasm was granular and showed cloudy swelling. In some areas, the epithelial lining showed desquamation and ulceration with degenerated cells and material filling the lumen. In some sections the tubules showed cystic dilation, while others contained pink-staining, homogeneous material and blood. No definite crystals of sulfadiazine could be found. The glomeruli showed no pathologic changes. The mucous membrane, lining the pelvis, was ulcerated, and occasional sulfadiazine crystals were found embedded in the wall or lying freely near the surface. The mucosa and submucosa in these areas were edematous, congested, and invaded with a purulent exudate. The pathology appeared to be confined to the convoluted tubules causing a toxic degeneration and necrosis rather than a mechanical blocking by the acetylsulfadiazine crystals. The lungs showed

marked edema and congestion with areas of bronchopneumonia. The leptomeninges surrounding the cerebellum were edematous and congested and showed slight evidence of a remaining inflammatory exudate. However, the leptomeninges of the cerebellum were intensely congested and showed a marked acute inflammatory cell reaction. No necrotic or inflammatory areas could be found in the liver.

CASE 6.—R. J., aged 21 years, was admitted to hospital in semicomatose condition. History of headache and marked prostration for two days. The patient was covered with petechiae from head to foot and numerous large areas of purpura. The spinal fluid done on admission contained 198 white blood cells of which 98 per cent were polymorphonuclears. No organisms were found on the smear. Red blood cells, 4,650,000; hemoglobin, 90 per cent; white blood cells, 7,200 with 70 per cent segmented forms, 15 per cent staff cells, 15 per cent lymphocytes. The blood culture was also taken on admission and was negative at forty-eight hours. The spinal fluid culture showed no growth. The patient was given sulfadiazine intravenously; plasma, saline, and glucose were administered, but in spite of the treatment, he became cyanotic and died eight hours after admission.

*Autopsy Findings.*—The face, neck, and entire body were covered with a purpuric rash and numerous petechiae. Some of the purpuric areas were 1 to 2 cm. in diameter, and cyanosis of the head and neck was prominent. The mesenteric lymph nodes were enlarged, and the thymus appeared larger than usual for the age of the patient. The lungs showed terminal edema and congestion. The spleen was enlarged and of firm consistency. There was a unilocular cyst measuring 5 cm. in diameter at one pole of the spleen. Both adrenals were enlarged, hemorrhagic, and degenerated. The adrenal arteries showed no evidence of thrombi. The right adrenal was triangular in shape and weighed 11 Gm. It measured 5.5 cm.  $\times$  4 cm.  $\times$  1.5 cm. The left adrenal was crescentic in shape and weighed 11 Gm. The measurements were 8 cm.  $\times$  3 cm.  $\times$  1 cm. The epicardium covering the left ventricle and auricle contained a moderate number of petechiae. There were a moderate number of petechiae on the serosal surface of the small intestine. The brain showed diffuse congestion of the superficial vessels. There was some flattening of the convolutions due to the edema of the brain. There was no frank purulent exudate covering the brain, but in a few areas the leptomeninges appeared as a thin whitish membrane. The cervical spinal cord and the brain stem showed only congestion.

*Histopathologic Findings.*—The microscopic picture of both adrenals was one of massive hemorrhage, causing almost complete destruction of both the medulla and cortex of both adrenal glands. It would appear that the medulla was more completely destroyed than the cortex by the diffuse hemorrhagic process. Special stains for bacteria in the adrenals were negative. Sections of the heart taken from the petechiae showed interstitial edema and extravasation of blood into the surrounding tissue. The superficial vessels of the brain were engorged, and the leptomeninges in the region of the cerebellum and cerebrum showed edema and congestion. In these areas there were a few collections of lymphocytes and plasma cells, but no evidence of any acute inflammatory exudate. Microscopic examination of the thymus showed the lymphatic elements rather prominent, considering the age of the patient. They were separated by wide strands of connective tissue and fat, showing some evidence of physiologic involution. The Hassal's corpuscles were few in number, and many were large and degenerated. A few were small, stained well, and were normal in appearance.

#### SUMMARY

1. Four cases of Waterhouse-Friderichsen syndrome occurring in adults are reported. In two of these the meningococcus was isolated as the etiologic agent.
2. A review of the literature reveals a total of 117 cases of Waterhouse-Friderichsen syndrome. Eighteen, including our four, occurred in adults.
3. One case of tubular degeneration of the kidneys due to sulfadiazine is reported.

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## JUVENILE DIABETES INSULIN SENSITIVITY

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THE young diabetic patient is frequently subject to periods of exacerbation and remission of the diabetic state. These periods occur independent of the dietary and in spite of the daily insulin medication. This period of exacerbation is most apt to appear during puberty and in some females during the menstrual cycle.

In the observed group the exacerbations were not due to dietary indiscretion or neglect of insulin. They were not associated with apparent infection or intoxication.

A similar picture of instability in diabetes with aggravation of the diabetic state was observed during pregnancy in the juvenile group. A profound change, characterized by unusual sensitivity to insulin, occurred about a half hour after expulsion of the placenta.

These periods of relative exacerbation and the periods of insulin sensitivity strongly suggested to us that the primary factor causing these changes might be extrapancreatic. The following clinical and experimental observations were conducted with this relationship in mind, and the results indicate that the anterior pituitary diabetogenic factor is responsible for certain exacerbations of the diabetic state.

### EXPERIMENTAL

The presence of the diabetogenic factor in the pituitary has been adequately and definitely demonstrated.<sup>1-7</sup> We have used anterior pituitary on mice and have found that the effect was to reduce sensitivity to insulin. The amount of insulin which would ordinarily kill a mouse in the Standard International Mouse Unit Test for Insulin will not be lethal for mice previously injected with anterior pituitary solution.

The hormone used in these experiments was made up as an acid alcoholic fraction of the anterior pituitary residue from which some of the usual sex-stimulating hormone and thyrotropic hormone had been removed by the method of Evans and had been salted out in the cold to form the hormone which has been named "Anterior Pituitary Diabetogenic Factor," prepared so that 1 c.c. was equivalent to 1 gram of the original gland substance.

Following the standard technique for the International Mouse Unit test for Insulin as modified, 20 Gm. mice were selected and fed a standard diet of whole wheat bread and milk for one week previous to the test. Three injections of one quarter of a c.c. (0.25 c.c.) each were given twenty-four hours, twelve hours, and two hours previous to the insulin injection. Approximately one mouse unit (0.02 clinical units) of standard insulin was given. The mice were immediately



placed at a temperature of 130° F. to produce a condition of strain. The animals were then examined every ten minutes at the end of one hour. The examination consisted of a determination for the condition of vertigo. Reaction is tested by injecting 50 per cent d-glucose solution intraperitoneally. There is recovery of the animals.

TABLE I  
EFFECT OF DIABETOGENIC ANTERIOR PITUITARY INJECTIONS AND 0.02 UNITS OF INSULIN ON MICE  
(SERIAL GROUP XX)

MOUSE NO.	WEIGHT (GM.)	TIME OF DEATH
1	20	Survived
2	19	Survived
3	18	Survived
4	19	2 hr. 10 min.
5	17	Survived
6	22	Survived
7	20	2 hr. 20 min.
8	23	Survived
9	17	Survived
10	22	Survived

TABLE II  
EFFECT OF INJECTION OF 0.02 UNITS OF INSULIN ON MICE  
(SERIAL GROUP XXI)

MOUSE NO.	WEIGHT (GM.)	TIME OF DEATH
1	23	2 hr. 10 min.
2	20	2 hr.
3	21	2 hr.
4	17	2 hr. 20 min.
5	19	2 hr.
6	21	2 hr. 10 min.
7	17	1 hr. 40 min.
8	16	2 hr. 20 min.
9	23	2 hr. 20 min.
10	21	Survived

Tables I and II are typical standardization groups. It will be noted that each group has within it approximately 20 per cent that are especially sensitive and approximately the same percentage that are insensitive.

#### CLINICAL

CASE 1.—R. B., white male, aged 13 years, height 59 inches, weight 95 pounds, developed diabetes at the age of 9. Onset was insidious. He was stabilized on 80 units of insulin daily which he received for three years. Early in March, 1941, he developed exacerbation of his diabetes requiring increased dosage of insulin to 260 units daily. On this dosage, blood sugar was 411 mg. per 100 c.c. at 10 A.M. and 95 mg. at 6 P.M. By March 30, the dose had to be further increased to 470 units daily. Blood sugar before breakfast was 480 mg., at 11 A.M. it was 354 mg., and at 1 P.M., 370 mg. The patient was admitted to the hospital on April 7 in precomatose state. He had been getting 600 units of insulin daily. On the day of admission he received 1000 units of insulin; blood sugar dropped to 35 mg., and he went into insulin shock. He has since been stabilized on 70 units of insulin daily, which dose he is receiving at the present time.

The period of maximum growth and sexual development is usually associated with activity of the anterior pituitary. The question arises whether the increased pituitary activity is responsible also for the acute exacerbation of the diabetic state.

This boy developed exacerbation of his diabetic state without any apparent extrinsic cause. He was then stabilized with daily insulin dosage approximating the level used before the acute exacerbation. It appears that the compensating mechanism has become disturbed during the acute phase.

CASE 2.—A. M., white male, aged 16 years, height 70 inches, weight 176 pounds, developed enuresis in 1938. He lost 60 pounds body weight, and on examination glycosuria was discovered. He was placed on an extremely low caloric diet and continued to lose weight. Glycosuria cleared on the low caloric diet, but after three months of strict dietary regulation he was given insulin because of his continued loss of weight. In 1939 he was given 5 units of insulin daily which controlled the glycosuria. During the next ten months he developed glycosuria and hyperglycemia, and insulin dosage had to be increased to 240 units daily. He was operated upon in September, 1939, for acute appendicitis and recovered. The sugar tolerance improved remarkably immediately after operation, and the insulin requirement was reduced to 10 units. He continued on this dosage for a short time after which the requirement was gradually increased to 100 units daily, the amount he is now receiving.

CASE 3.—S. R., white female, aged 22 years, height 61 inches, weight 130 pounds, developed diabetes at the age of 18. Between 1936 and 1941 she took 130 units of protamine insulin before breakfast every day. During this time she complained of difficulty with menstruation, suffering from cramps and nausea. Periods occurred between thirty-six and thirty-seven-day intervals and lasted six or seven days. Just prior to the period, the patient developed acidosis and coma. Insulin dosage was increased to 200 units daily at the beginning of the period. This controlled the exacerbation. Immediately after cessation of the period, the regular dose of 130 units daily would control the diabetes until the next period. With this change in insulin dosage during the menstrual period, patient has avoided hospitalization for the past three years.

The regularity of the occurrence of the exacerbation at the menstrual cycle indicates a close relationship and a lack of the normal balancing factors of posterior pituitary against anterior pituitary.

Certain similarities have been noticed in the manner of onset of juvenile cases. This is the normoglycemic diabetic state which is often mistaken for renal diabetes. In our experience we have not found renal diabetes to be an entity. In all cases observed the normoglycemic state (renal diabetes) gave way to frank and severe diabetes mellitus with hyperglycemia.

Cases 4 and 5 following are such typical case histories. Case 6 is now in the normoglycemic diabetic state. Insulin therapy is being utilized as a possible prophylactic against the development of diabetes mellitus.

CASE 4.—F. E., white male, aged 29 years, height 73 inches, weight 155 pounds, developed diabetes at the age of 14. For four years prior to the onset of the diabetic state, he had been subject to repeated attacks of vomiting and acetoneuria without glycosuria. Blood sugar on admission was found to be 500 mg. He was maintained on a low caloric diet and 15 units of insulin daily. After six months' observation starvation blood sugar dropped to 130 mg. per 100 c.c. There was no sugar in the urine. Insulin therapy was stopped. After a period of eight months without insulin, patient again developed glycosuria and acetoneuria. Insulin therapy was again instituted, the patient receiving 80 units of insulin daily. This dosage has been maintained for the past fourteen years.

CASE 5.—G. K., white female, aged 22 years, height 66½ inches, weight 123 pounds, was found to have diabetes in 1935 when glycosuria was discovered. At this time blood sugar level was 140 mg. Glycosuria persisted although the blood sugar level remained about the same. She was placed upon a diet containing 250 carbohydrates, 100 proteins, and 120 fats. No insulin was given. In April, 1936, she was admitted to the hospital in diabetic coma from which she recovered and was placed upon crystalline insulin, 45 units daily. In February, 1942, patient developed nausea and vomiting with the menstrual period. This was accompanied by increased glycosuria. It was necessary to increase the insulin dose by 40 to 100 units daily. The increased insulin did not relieve the nausea or vomiting. During the period of vomiting, blood sugar levels ranged up to 185 mg., indicating that the nausea and vomit-

ting were not due to the glycosuria, but that the exacerbation of the diabetes and the nausea and vomiting were part of the menstrual disturbance.

It is of special interest in this case that there was a period of six months during which the blood sugar level was 140 mg. or less; yet there was persistent glycosuria. The patient received no insulin during this time. This may be considered a normoglycemic diabetic state. Yet in six months she developed a diabetes which required 45 units of insulin daily to control and more recently an acute exacerbation at the menstrual cycle which required from 95 to 145 units of insulin daily to control.

In view of our experience with the above group of cases, it may be observed that the normoglycemic state has been followed by a definite severe active diabetes mellitus. While they could be maintained for a period of months, there followed in this group exhibiting glycosuria a definite development of frank diabetes mellitus requiring permanent insulin therapy. It was felt that in the following patient who appeared to be a normoglycemic diabetic an attempt should be made to prevent the occurrence of frank diabetes which developed in the previous group. It was our opinion that small to moderate doses of insulin utilized over a period of time might prevent the occurrence of frank diabetes mellitus.

CASE 6.—B. H., white male, aged 7 years, height 48½ inches, weight 53 pounds, was found to have glycosuria. This was present for a month before admission to the hospital. On admission, urine sugar was 1½ per cent, blood sugar 118 mg. per 100 c.c. Twenty-four hours after administration of 20 units of protamine insulin, blood sugar was 78 mg.; urine showed 1½ per cent sugar. Observation of the patient revealed mental dullness, lack of growth, inanition, and asthenia. In May, 1941, insulin therapy was instituted, 20 units twice a week. Since this time he has gained 25 pounds. His mental condition has changed for the better. He has become social. The glycosuria is now consistently less than 0.5 per cent. Blood sugar in August, 1941, was 65 mg.; in December, 1941, 65 mg.; in April, 1942, 65 mg. The glycosuria was present at the time of these blood tests. There has been an increase in growth.

This improved general condition appears to us to be adequate incentive to justify persistence in this attempt at diabetic prophylaxis.

#### PREGNANCY IN JUVENILE DIABETES

Profound changes occur in the pituitary during pregnancy. These changes are mainly concerned with hyperplasia of the pituitary and an overflow of anterior pituitary-like substance in the urine. Furthermore, immediately following delivery of placenta, other changes occur which indicate a balancing of the overactive pituitary. In this group the change is demonstrated by the development of a hypersusceptibility to insulin. This state is important inasmuch as the sensitivity to insulin may become so great that the average dose for that individual may cause fatal insulin shock (Case 3).

The importance of this oversensitivity indicates that in the diabetic patient there is a loss of balance between the diabetogenic and insulin factors. If the origin of the factor producing the sensitivity could be demonstrated, a contribution would be made to diabetic therapy.

The following five cases illustrate the changes in the juvenile type of diabetic patient during pregnancy.

CASE 7.—G. P., white female, aged 17 years, height 61 inches, weight 139 pounds. Amenorrhea had persisted for one month. The blood sugar time curve (blood sugar taken every two hours for twenty-four hours) showed no change from the curve previous to pregnancy. It showed a high morning level which rapidly fell and reached its lowest point at 8 P.M., which level persisted at 4 A.M. and rose to a high morning level of 300 mg., lasted for a short time only and was reduced to the average level of 150 mg. by the morning insulin. After twenty-nine weeks of pregnancy, it was noted that the patient had become sensitive to early morning insulin shock, although the dose was reduced from 60-0-20 to 35-0-20. In spite of this low morning sugar level, the patient showed a rise of over 300 mg. in the period of 4 to 10 P.M. After this, the blood sugar returned to a reasonable level of 250 mg., the average level for around this time being about 250 mg. This indicated that a change had occurred and more insulin was required. By the thirtieth week 75-0-35 units of insulin were

required, and in the thirty-seventh week 75.0-30. The baby was born in the thirty-eighth week. Blood sugar of the child eight hours after birth was 70 mg.

CASE 8.—H. N., white female, aged 17½ years, height 64 inches, weight 116½ pounds, had had diabetes for seven years. She was stabilized on an insulin dosage of 65 units daily. Diet was largely qualitative, but the amount of carbohydrate was controlled at 275 to 300 Gm. During pregnancy the insulin requirement was unchanged. She delivered spontaneously at term. The child weighed 6 pounds, 7 ounces. The first day of post partum the full insulin dose of 65 units was given. This was repeated on the second day. No consideration was given the increase in insulin sensitivity occurring post partum in juvenile cases. Eight hours after receiving the second portion of the 65 unit dose (30.0-35) on the second day post partum, the patient went into extreme shock. The blood sugar was 17 mg. The patient died in spite of intravenous glucose.

CASE 9.—D. M., white female, 18½ years old at the time of pregnancy, height 62 inches, weight 140 pounds. Onset of diabetes was insidious at the age of 13. She had been standardized on 100 units of protamine insulin daily with diet of 240 Gm. carbohydrates. She delivered spontaneously after an uneventful pregnancy. The child was male, weighing 6 pounds. Infant died of pneumonia two days after birth. In this case the insulin requirement was checked carefully and found to range from 28 to 40 units during the first five days post partum. Patient meanwhile was on a full diet. During the next month the insulin requirement showed a gradual rise to 100 units and has remained at this level for the past two years.

CASE 10.—L. P., white female, 25 years old at the time of pregnancy, height 61 inches, weight 125 pounds. Onset of the diabetes occurred at the age of 17. Although there were no obstetrical indications from an anatomic standpoint, a cesarean was performed as the method of choice of the obstetrician. This patient had been established on 60 units of protamine insulin daily for seven years previous to pregnancy. On the day before the operation she had received her usual dose of 60 units. The child was born at 3:50 A.M., a normal male weighing 13 pounds, 14 ounces. Patient's blood sugar at 1:45 A.M. was 100 mg. and at 3:15 A.M. 56 mg. The baby's blood sugar at birth was 50 mg. and the blood sugar of cord at birth 100 mg. At 4:10 A.M., patient's blood sugar was 117 mg.; at 8:00 A.M., 277 mg.; at noon, 130 mg.; and at 8:00 P.M., 73 mg. During the twelve days after delivery, the insulin requirement fell at times to as low as 25 units a day. On discharge, she was receiving 45 units of insulin daily.

CASE 11.—M. E., white female, aged 27 years at the time of pregnancy, height 66 inches, weight 130 pounds. Onset of the diabetes occurred at the age of 23. She had been stabilized on 75 units of crystalline insulin daily, divided into doses of 40.0-35. She received this amount of insulin daily throughout her pregnancy. She had an uneventful pregnancy and spontaneous delivery of a male child weighing 6 pounds, 15 ounces. For fifty-two days following delivery, insulin requirement was reduced to 50 units a day in doses of 25.0-25 of the same insulin. Carbohydrate intake was the same before and after delivery.

#### DISCUSSION

The insulin dosage of this group of juvenile type of diabetic patients had been regulated to a relatively constant dosage ranging between 60 and 100 units daily. While the insulin dosage had to be increased during the pregnancy of some of the patients, in every case there was obvious evidence of a marked sensitivity to insulin during and immediately following parturition. The requirement for insulin was reduced at least 15 units daily and then showed a gradual return to the usual dose for the individual within a period of ten days.

It is of interest that where blood sugar could be adequately controlled, immediately following parturition the blood sugar rose as in a postprandial rise. It is important that this postprandial type of rise not deceive the physician into giving more insulin as in Case 7. In this case although the blood sugar was

brought within 75 to 180 mg. for the four hours previous to delivery and was 150 mg. at the time of delivery, it rose to 300 mg. immediately after delivery. Additional insulin was administered. It was found that the patient had developed an increased sensitivity to insulin, and a dose as low as 10 units brought about insulin shock. For a week following parturition, the insulin requirement was 15 units daily and then gradually had to be increased to her parturition dose.

In Case 8 the insulin dose had been established at 65 units daily for over a six-year period. Unfortunately, immediately post partum the insulin-sensitive state was not recognized. Patient was given her regular dose of insulin. This resulted in insulin shock which was not controllable, and she died with a blood sugar level of 17 mg.

#### SUMMARY

Clinical case reports are presented describing the exacerbation of the diabetic state in association with what may be considered increased anterior pituitary activity. In the early stages of the increased activity there is cessation of the diabetic state for a period of time running into many months. In this group, the normoglycemic type is presented showing a periodic glycosuria and then the development of a definite and permanent diabetes mellitus.

It was felt that the use of insulin in the normoglycemic might serve as a prophylactic measure in prevention of the permanent diabetic state.

Clinical case reports of five of the juvenile type diabetic patients observed before and throughout their pregnancies are presented. Each case shows dramatic insulin sensitivity during the immediate post-partum period. The dose of insulin to which the patient had been regularly accustomed for a period of years becomes a toxic and even fatal amount. It is possible and even probable that the mechanism here is the antianterior pituitary diabetogenic factor. The authors feel that this factor may come from the posterior pituitary.

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# INEFFECTIVE PENICILLIN CHEMOTHERAPY OF ARTHRITIC RATS INFECTED WITH PLEUROPNEUMONIA-LIKE ORGANISMS\*

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## INTRODUCTION

POWELL and Jamieson<sup>1,2</sup> have recently reported on the high degree of chemotherapeutic effectiveness of penicillin in white mice infected with normal and sulfonamide-fast pneumococci and staphylococci. In these tests penicillin was found to bring about the "cure" of pneumococcus-infected mice, irrespective of whether the infections were due to normal or sulfonamide-fast organisms. Furthermore, penicillin was found to save the lives of approximately twice as many staphylococcus-infected mice as was accomplished by sulfathiazole under the same conditions. The implications of these and other laboratory and clinical results that cannot be reviewed here add promise to the chemotherapeutic importance of penicillin.

In the present report the results of penicillin chemotherapy of rats infected with pleuropneumonia-like organisms are presented.

## EXPERIMENTAL

As experimental animals, white rats of about 100-gram weight were used.

The pleuropneumonia-like culture was isolated in this laboratory from a spontaneous joint infection in a laboratory rat. This animal showed a greatly swollen right foreleg, particularly at the radiocarpal joint, and the initial culture was obtained by aspirating a drop of fluid from this joint with a syringe and culturing in beef infusion broth enriched with 30 per cent ascitic fluid. On incubation at 37° C. for 24 hours, this and subsequent cultures on this medium showed marked turbidity, and after an additional 24 hours' incubation the growth generally settled as a deposit of flakes in the bottom of the tube. Microscopically the culture showed either small gram-negative bacilli or gram-negative debris or both. This culture did not grow in plain broth or agar.

The optimum technique for reproducing arthritis in normal rats consisted in injecting 0.5 c.c. of 24-hour ascitic fluid broth culture intravenously. Subcutaneous, intramuscular, and intraperitoneal routes of injection resulted in irregular appearance of arthritis. The same appeared to be true of intravenous doses of less than 0.5 c.c. During the third, fourth, and fifth days after injection, a progressive polyarthritis developed, in which there was a fusiform swelling of the toes, and progressive swelling of leg joints, the skin over these becoming tight and glistening and red or purplish. Within a few days, many of the infected rats showed a hemorrhagic deposit and edema about the nose and sometimes a conjunctivitis. An occasional rat had neurologic symptoms. Rats infected as described and receiving no chemotherapy died, generally in about

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two or three weeks, and for some days before death were quite prostrated and helpless. Certain hopelessly crippled rats with massive lesions were sacrificed.

The infections in general were similar to rat infections described by Findlay, Mackenzie, MacCallum, and Klieneberger,<sup>3</sup> and those reported previously by Collier.<sup>4</sup> They were more rapidly progressive than similar experimental mouse infections reported in the last two or three years, such as those of Sabin and Warren,<sup>5</sup> and resembled closely those recently described by Preston.<sup>6</sup>

The penicillin used was similar to the partially purified material referred to in previous reports<sup>1, 2</sup> and that reported upon by Schmidt and Sesler.<sup>7</sup>

In addition to control animals which received no treatment, rats treated with gold sodium thiomaleate (myochrysine) were used as controls for the different groups of penicillin-treated rats. Results in such groups of infected rats would reveal (1) whether a new drug had any demonstrable chemotherapeutic potency and (2) whether this action compared favorably with myochrysine, an agent which is known to have a curative effect on this type of experimental arthritis.

TABLE I

COMPARATIVE CHEMOTHERAPEUTIC RESULTS IN RATS OF 100 GRAMS WEIGHT RENDERED ARTHRITIC WITH PLEUROPNEUMONIA CULTURE

DRUG	NUMBER OF RATS	DRUG DOSES PER RAT X NUMBER DOSES	RESULTS
Penicillin	2	100 units x 9	DD
	4	800 x 9	SDDD
	4	8000 x 5	DDDD
	4	500 x 6	DDDD
	2	200 x 4	DD
	2	200 x 5	DD
	4	400 x 6	DDDD
Myochrysine	1	10 mg. x 3	D
	1	5 x 3	S
	4	5 x 3	SSDD
	4	5 x 3	SSDD
	4	3 x 3	SSDD
	4	2 x 1	SSDD
Controls	2		DD
	3		DDD
	4		DDDD
	4		DDDD
	4		DDDD

All rats marked S survived with either no symptoms or early mild arthritic symptoms which subsided rapidly. All penicillin and control rats marked D died of progressive arthritis, or in a few instances when helpless and moribund were sacrificed. All myochrysine rats marked D died with no arthritis, probably of drug toxicity.

Twenty-two rats were treated with a variety of doses of penicillin, as shown in Table I. The drug was given by different routes, intravenously, intraperitoneally, intramuscularly, and subcutaneously, without much detectable effect. In a few instances development of arthritis appeared to be slowed somewhat, and a single penicillin-treated rat survived without developing arthritis. On the whole, however, penicillin appeared to exert no practicable degree of curative action. The rats died of progressive arthritis, hemorrhagic pulmonary edema, and terminal diarrhea.

Eighteen rats were treated with a variety of doses of myochrysine, as shown in Table I. The drug was given either intramuscularly or subcutaneously.

Seven of these 18 rats survived without appearance of arthritic lesions, or developed fusiform swelling of the toes which rapidly subsided. The eleven rats which did not survive on gold therapy showed no arthritis at the time of death and appeared to have died because of drug toxicity. The toxicity of myochrysine seemed to be somewhat unpredictable, since several deaths occurred on 2 or 3 mg. doses while 5 mg. in some instances did not appear to be toxic. From the results recorded it is apparent that doses of myochrysine less than 2 mg. might have been equally effective.

Seventeen infected control rats all died of progressive arthritis.

#### CONCLUSIONS

It appears from the foregoing results that:

1. Penicillin has little or no chemotherapeutic effectiveness in infectious rat polyarthritis of the type described.

2. Myochrysine, as a control drug, was chemotherapeutically effective but quite toxic in the doses used.

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# THE OCCURRENCE OF MEMBERS OF THE GENUS *SALMONELLA* IN INHABITANTS OF STATE HOSPITALS OF THE GREATER CHICAGO AREA\*

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THE most extensive report on the occurrence of *Salmonella* types in the United States is that of Bornstein.<sup>1</sup> The greatest number of strains belonging to the genus *Salmonella* was, however, studied in the National *Salmonella* Center by Edwards and Bruner.<sup>2</sup> These authors state that the most frequent *Salmonella* types found in this country in man are *S. schottmülleri*, heretofore called *S. paratyphi B*, and *S. typhimurium*, designated in the past as *S. aertrycke*. The conclusions of the New York group of investigators, headed by Bornstein,<sup>3</sup> give similar results for the eastern United States. This center reports 40 per cent of *Salmonella* strains belonging to other types than *S. schottmülleri* and *S. typhimurium*. Also Borman et al.<sup>4</sup> of Connecticut, and Gaulton and Quan<sup>5</sup> of Florida describe a series of less common *Salmonella* strains among their findings.

It may be concluded therefore that the predominant members of the genus *Salmonella* in man are *S. schottmülleri* and *S. typhimurium*, but other types will be encountered in many cases.

The laboratory diagnosis of less common *Salmonella* strains involves the use of a long series of biochemical and serologic reactions, the latter known routinely as "*Salmonella* typing." While the determination of a typical *S. paratyphi*, *S. schottmülleri*, or *S. typhimurium* can be easily accomplished even in a small laboratory, the exact typing of other *Salmonella* species and varieties involves the uses of slide agglutination tests with 23 "O" sera and tube agglutination tests with sometimes as many as 36 "H" sera. Hence there is a necessity for special laboratories to perform this task.

Under the auspices of the Division of Laboratories of the Department of Public Health of Illinois, a special service was established in Illinois state hospitals to examine material for organisms causing enteric diseases, such as *Salmonellae*, *Eberthellae*, *Shigellae*, and parasites. In September, 1942, a central enteric laboratory was established for the identification of organisms isolated in the local laboratories of state hospitals.

The "local bacteriologist" streaks the stool specimens to a series of plates: Wilson and Blair in the modification of Hajna and Perry,<sup>6</sup> Leifson's desoxycholate-citrate medium,<sup>7</sup> Difco S.S. agar, Difco eosine-methylene blue plate, and either MacConkey's medium in the modification produced by Difco, or Leifson's desoxycholate plate. A tube of selenite-F of the Baltimore Biological Lab-

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*S. dare-es salaam* has never been found in America. It differs from most *Salmonella* types in its ability to liquefy gelatin. The strain here observed came from a 63-year-old factory worker, newly admitted to a state hospital. It could not be recovered from the stool specimen on re-examination after four weeks.

*S. panama*, an organism often encountered, was found in a 53-year-old former food handler when examined on admission. The feces became negative within two months.

*S. pullorum* was isolated from six cases of diarrhea. This infection is of special interest, being a very rare human incidence of *S. pullorum*. Edwards and Bruner<sup>2</sup> reported only two cases in man. Three other cases were mentioned by Edwards in a personal communication. One human case was found by Borman et al.<sup>4</sup>

The cases observed in the present survey appeared in two groups in the same state hospital.

The first group included two cases that appeared on different wards but at the same time. One patient was a 48-year-old former housewife who had been hospitalized for thirteen years. She had "dysentery" of unknown origin seven years ago. In March, 1943, she became ill with diarrhea. Light-colored liquid stools with much mucus, slightly raised temperature, and abdominal pain were recorded. *S. pullorum* was isolated from the stools during the first days of sickness. The patient was treated with sulfasuxidine and recovered within three weeks.

The other case appearing at the same time was a 72-year-old factory worker who had been hospitalized for seven years. When she became ill with diarrhea, and had light-colored liquid stools with much mucus, slight abdominal pain but without fever, *S. pullorum* was isolated from the stool specimens. She also received sulfasuxidine treatment. Frothy liquid stools were observed for four weeks. In May, 1943, no symptoms were recorded except mucorrhea. The stool specimens did not reveal *Salmonella*. In June, 1943, the patient became ill with bronchopneumonia and expired. The autopsy did not show pathology of the intestines or gall bladder. No *Salmonellae* were isolated from the cadaver.

The second group of *S. pullorum* infections consisted of four cases and occurred in August and September, 1943.

Two of these cases suffered from diarrhea repeatedly. The first of them, a 59-year-old patient, had diarrhea of unknown origin in February, 1941, April, 1942, and June, 1942. An attack of diarrhea in August, 1943, showed claylike, mucous, liquid stools. No general symptoms were noticed. *S. pullorum* was isolated during the first week of illness. The patient recovered under sulfasuxidine treatment within two weeks.

The other patient whose history showed repeated attacks of diarrhea was 69 years of age. She had dysentery-like diarrhea with negative bacteriologic findings in December, 1942, and February, 1943. In August, 1943, soft, mushy stools were observed, with much mucus. There was no fever and no pain. *S. pullorum* was isolated from the first diarrheic stool specimen. The disease disappeared within two weeks on sulfasuxidine treatment.

The last two patients of this group, aged 50 and 78 years, had diarrhea with liquid, light-brown stools and considerable mucus, some pain in the left hypogastric region, and slightly raised temperature. When *S. pullorum* was isolated from the stools, treatment with sulfasuxidine was introduced. The diarrhea ceased within a week and the patients were considered recovered in twenty-one days.

The source of the *S. pullorum* infection could not be discovered. Because of the technical difficulties encountered in the isolation of the very fastidious *S. pullorum*, it may be presumed that several cases remained undetected. The hospital does not serve raw meat or eggs to inmates. Because of the appearance of four cases on the same ward, contact infection has to be assumed. The history of three of the six patients with *S. pullorum* diarrhea showed previous abdominal disturbances. It is impossible to attempt to answer the question as to whether a chronic *S. pullorum* infection was present in these cases or if this Salmonella found a fertile soil in the intestines altered by a pathologic process of a different etiology.

*S. anatum* and *S. newington*, often isolated from man and animals, were each present in one chronic carrier who acquired this status during outbreaks of such infections in state hospitals in previous years. In addition, two chronic human carriers of *S. anatum* were isolated among new admissions to state hospitals.

*S. senftenberg*, an organism frequently causing enteritis in man, was observed in two temporary carriers during a survey and in one case of a dysentery-like diarrhea the origin of which could not be traced. This diarrhea was successfully treated with sulfasuxidine and ceased within a week.

The last group of Salmonellae, called "F" or "Further groups," causes the greatest difficulty in diagnosis. While 9 "O" sera are sufficient to classify roughly a member of the groups "A" to "E," 14 "O" sera have to be employed for the diagnosis of one of the 23 Salmonella types belonging to this category. Many laboratories do not type members of the "F" group at all. The observation of the following three strains, however, demonstrates the necessity to examine organisms belonging to this group.

*S. kirkee* was described by Bridges and Dunbar,<sup>11</sup> but has not been reported in the United States. In this survey a strain was isolated from a 56-year-old factory worker recently admitted to a state hospital. Only one stool specimen showed this organism. The patient died of tuberculosis three months later. No significant findings were described in the autopsy protocol, and no Salmonella was isolated from the cadaver.

*S. ballerup* of Kauffmann and Miller<sup>12</sup> has never been reported in this country. According to personal communications from Drs. Edward, Fulton, and Seligman, and as far as can be ascertained from the literature, this is the first instance since the original description of this organism that *S. ballerup* has been identified.

The strain was isolated from a 36-year-old housewife when admitted to a state hospital. She denied having diarrhea or fever. The proctoscopic findings were negative. The patient ceased to be a carrier within two weeks.

*S. ballerup* here isolated contains Vi antigens and exhibits typical V-W variation.

*S. kentucky* was first described by Edwards<sup>19</sup> from fowl enteritis. Olitzki<sup>20</sup> isolated a "var. *palestina*" in camel and man characterized by the fermentation of glycerol and no action upon m-inositol. Borman et al.<sup>4</sup> found this *Salmonella* in a human carrier.

The strain observed in this survey came from a 58-year-old factory worker and was isolated on admission to a state hospital. It was glycerol-negative and m-inositol positive, thus giving the reactions of the original type of Edwards.

The patient did not show *Salmonella* on re-examination after one month.

#### DISCUSSION

During the period of one year, 65 *Salmonella* strains were isolated from 64 patients and employees of state hospitals of the greater Chicago area. While 47 strains belonged to more frequently seen types, as *S. schottmülleri*, *S. typhimurium*, *S. oranienburg*, *S. bareilly*, *S. monteideo*, *S. choleraesuis* var. *kuntzendorf*, *S. enteritidis*, *S. panama*, *S. anatum*, and *S. senftenberg*; 15 strains represented less common types as *S. schottmülleri* animal strain, *S. schottmülleri* var. *java*, *S. newport* var. *puerto rico*, *S. pullorum*, *S. newington*, and *S. kentucky*. Finally three types were isolated in the United States for the first time, as *S. dar-es-salaam*, *S. kirkee*, and *S. ballerup*.

*Salmonellae* were frequently found in symptomless carriers. It deserves attention that ten of the twenty-six carriers were food handlers by profession. The great number of such carriers among newly admitted state hospital patients during the last months could be linked, perhaps, with the increasing consumption of fowl and uninspected meat.

*S. typhimurium*, *S. monteideo*, *S. newport* var. *puerto rico*, *S. enteritidis*, *S. dar-es-salaam*, *S. panama*, *S. senftenberg*, *S. kirkee*, *S. kentucky*, and *S. ballerup* caused only transitory carrier status. *S. schottmülleri* and its varieties, *bareilly*, *anatum*, and *newington*, were isolated from persons harboring such organisms for more than three months. *S. oranienburg* was found in both a temporary and a chronic carrier. *S. pullorum* and *S. choleraesuis* var. *kuntzendorf* were isolated only from cases of disease.

As to the propagation of *Salmonellae* in human beings, man-to-man transfer had to be assumed in infections observed with *S. schottmülleri* animal strain, *S. pullorum*, *S. typhimurium*, *S. choleraesuis* var. *kuntzendorf*, *S. oranienburg*, and *S. monteideo*. Food as the source of infection could be traced in one outbreak of *S. typhimurium* diarrhea. The origin of the first cases of enteritis due to *S. choleraesuis* var. *kuntzendorf*, *S. pullorum*, *S. monteideo*, and *S. senftenberg* could not be discovered, but food was suspected.

The clinical picture of the "Salmonella disease" was the dysentery-like type of Hormaeche<sup>21</sup> with or without fever and abdominal pain. The diarrhea caused by *S. pullorum* showed a striking amount of mucus. The prognosis of the cases here observed was good. Recovery came within two to three weeks on the average. *S. pullorum*, *S. oranienburg*, *S. senftenberg* and *S. monteideo* infections were successfully treated with sulfasuxidine; *S. choleraesuis* var. *kuntzendorf*, with sulfathiazole. Cases due to *S. typhimurium* were more resistant to sulfa treatment. The sulfa treatment of chronic carriers of *S. schottmülleri*, *S.*

*anatum*, *S. bareilly*, *S. newington*, and *S. oranienburg* was a failure. The small number of observed cases, however, does not permit definite conclusions. It seems to be necessary to try also other drugs than were used in these cases (sulfathiazole and sulfasuxidine).

The so-called "Kiel doctrine" distinguished *Salmonellae* pathogenic for man from *Salmonellae* afflicting animals. The "Montevideo doctrine," however, recognizes that all *Salmonellae* are potential pathogens for both man and animals.<sup>1</sup> This doctrine emphasizes that age, nutrition, general resistancy, and similar factors are of great importance to the development of *Salmonella* infections. The results of the survey here described definitely indicate that aged people, suffering of mental diseases and consecutive malnutrition, are more liable to become cases and carriers of *Salmonellae* than healthy individuals. Whether the invading *Salmonella* type is more "human" or more "animal" adapted does not seem to play an eminent role.

TABLE I

## DISTRIBUTION OF SALMONELLA TYPES IN STATE HOSPITAL PATIENTS OF THE GREATER CHICAGO AREA

From Oct. 1, 1942, to Oct. 1, 1943

SALMONELLA TYPE	NUMBER OF CASES	NUMBER OF CARRIERS	TOGETHER
<i>schottmülleri</i>	0	4	4
—animal strain	0	4	4
—var. <i>java</i>	0	2	2
<i>typhimurium</i>	10	8	18
<i>choleraesuis</i> var. <i>kuntzendorf</i>	2	0	2
<i>oranienburg</i>	2	2	4
<i>bareilly</i>	0	1	1
<i>montevideo</i>	5	4	9
<i>newport</i> var. <i>puerto rico</i>	0	1	1
<i>enteritidis</i>	0	2	2
<i>dar-es-salaam</i>	0	1	1
<i>panama</i>	0	1	1
<i>pullorum</i>	6	0	6
<i>anatum</i>	0	3	3
<i>newington</i>	0	1	1
<i>senftenberg</i>	1	2	3
<i>kirkee</i>	0	1	1
<i>ballerup</i>	0	1	1
<i>kentucky</i>	0	1	1
Total	26	39	65

## SUMMARY

From 13,000 stool specimens of inhabitants of state hospitals of the greater Chicago area, 65 strains of *Salmonella* belonging to 19 types of this genus were isolated. The occurrence of the respective types is discussed, and the observation of less common *Salmonellae*, as *S. schottmülleri* animal strain, *S. schottmülleri* var. *java*, *S. newport* var. *puerto rico*, and *S. kentucky*, is recorded. An unusual epidemic of *S. pullorum enteritis* in man is discussed. The first isolation of *S. dar-es-salaam*, *S. kirkee*, and *S. ballerup* in the United States is described.

The authors are very much indebted to Dr. P. R. Edwards, Director of the National Salmonella Center, Agricultural Experiment Station, University of Kentucky, Lexington, Ky., for constant help and advice in their work. Dr. Edwards identified all less common and many of the frequently found *Salmonella* types here mentioned.

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## NUTRITIONAL STATUS OF STAPHYLOCOCCUS AUREUS AS INFLUENCED BY PROFLAVINE\*

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THE newer approach to the problem of chemotherapy lies in the determination of altered bacterial nutrition or metabolic status caused by an anti-septic. The exclusion principle of Fildes<sup>1</sup> has given impetus to investigations of this type. Burke and his co-workers<sup>2</sup> first demonstrated that *Staphylococcus albus* develops resistance to acridines. In their experience, the adaptation to the dye was temporary and disappeared when the organism was grown on dye-free agar. The work of McIlwain<sup>3</sup> has partially disclosed the nature of the mechanism of adaptation in these bacteria. Using *Bacterium coli* and *Streptococcus hemolyticus*, he found two types of material which were needed for growth in the presence of the acridines but were not needed in normal bacteria. Type 1 was nucleotides and Type 2 was a concentrate of amino acids for which phenylalanine could be partially substituted. Type 1 compounds formed complex salts with acriflavine components, and McIlwain considered that the inhibitors inactivate enzyme systems, of which Type 1 compounds are an essential part and Type 2 compounds are substrates or products, some of which can be replaced by hydrogen carriers.

Recent revival of interest in acridines has been due largely to war medicine and to a report from a British Medical unit operating in North Africa.<sup>4</sup> The present investigation was started with the objective of extension and improvement in the acridine type of chemotherapy.

**Procedure and Results.**—The chemicals being tested for inhibition of the bacteriostatic and bactericidal action of proflavine were weighed into 10 c.c. amounts of standard beef extract broth. Each tube was subsequently inoculated with 0.1 c.c. of a 1/10 dilution of a 22- to 26-hour culture of *Staphylococcus aureus* No. 209. The tubes were incubated 18 hours at 37.5° C. The number of bacteria per c.c. was determined by the serial dilution plate count method. As an added check, a system for determination of density using reflection methods was employed. Each set contained the experimental tube, the control, and the tube with the experimentally used chemicals. The latter tube was not inoculated. This made it possible to dilute the experimental tube with media and compare with the control plus an equal volume of incubated chemicals, the latter being used as the 100 setting and media as the zero. For purposes of listing, the chemicals tested are divided into five series: intermediary carbohydrate metabolic products, amino acids, vitamins, purines and pyrimidines, and miscellaneous. A total of 30 experiments, 1000 serial dilution counts, and 2500 media tubes were needed to obtain the results.

**Carbohydrate Intermediate Metabolic Products.**—These chemicals were tested at concentrations up to 0.5 mg. per c.c. The proflavine concentration

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unless otherwise stated was 1 in 200,000 which is that concentration which completely inhibits the growth of *Staphylococcus aureus* under our experimental conditions. All of these chemicals possessed some activity with the exception of dihydroxyacetone, glucuronic acid, and fructose. Table I shows the results obtained with various intermediary products of carbohydrate metabolism. It is felt that their activity is not due to chemical interaction with the acridine used, proflavine. It is possible to conceive of direct chemical interaction between the amino groups of the diamino-acridine and the aldehyde group of glucose, but it is difficult to imagine such action occurring between a simple acid like acetic and the proflavine molecule. It is highly probable that the action is the direct result of mass action forces on enzyme systems inhibited by the acridines. Only pyruvic acid proved effective at concentrations of 0.1 mg. per c.c.

TABLE I

THE INFLUENCE OF INTERMEDIARY CARBOHYDRATE METABOLITES ON PROFLAVINE ANTISEPTICS  
(Averages for four sets)  
(Values expressed as number of bacteria per c.c.)

0.5 MG./C.C. COMPOUND	0.05 MG./C.C. PROFLAVINE +COMP.	0.05 MG./C.C. PROFLAVINE	CONTROL	COMPOUND
Glucose	32,400,000	47,000	189,000,000	
Glucose	250,000*	47,000	189,000,000	23,760,000*
Acetic Acid	40,000,000	117	36,000,000	40,000,000
Acetic Acid	1,296,000	6,480	18,000,000	1,296,000
Malic Acid	83,400,000	117	90,000,000	
Succinic Acid	48,000,000	117	90,000,000	110,000,000
Fumaric Acid	28,200,000	117	90,000,000	
Pyruvic Acid	39,000,000	96	36,000,000	40,000,000
Pyruvic Acid	75,000,000	120	25,000,000	
Lactic Acid	34,200,000	96	36,000,000	
Gluconic Acid	500,000	96	36,000,000	

\*10.0 mg./c.c.

*Amino Acids.*—The following amino acids were tested at concentrations of 0.5 mg. per c.c.: phenylalanine, tyrosine, histidine, lysine, arginine, cysteine, glutamic acid, threonine, glycine, serine, tryptophan, leucine, isoleucine, valine, alanine, methionine, aspartic acid, proline, and hydroxyproline. All were tried as inhibitors of proflavine in concentration up to 0.5 mg. per c.c. Of the series, phenylalanine and tyrosine were weakly positive, while cysteine, histidine, and glutamic acid were strong inhibitors. Table II shows the results obtained with the positive agents. Glutamic acid as a substrate of an adenine-containing enzymatic system fits into the scheme which will be presented; the other amino acid inhibitors of proflavine do not. Again, direct chemical interaction is improbable as the positive acids contain a representative of the dicarboxylic amino acids, of the basic amino acids, and of the neutral amino acids. Further, aspartic acid, a dicarboxylic amino acid, is negative, while glutamic acid of the same group is positive. Histidine, a basic amino acid, is positive, while arginine and lysine, other basic amino acids, are negative. These facts make it improbable again that direct chemical interaction is the explanation. None of the amino acids tested was active at 0.1 mg. per c.c.

*Vitamins.*—The vitamins tested included nicotinamide, thiamine, inositol, pyridoxine, pantothenic acid, riboflavin, biotin, and p-aminobenzoic acid. These



vitamins were negative without exception. The phosphorylated form of thiamine, cocarboxylase, was weakly positive at a concentration of 0.5 mg. per c.c.

*Purines and Pyrimidines.*—The members of this series included: adenine, guanine, guanosine, uracil, thymine, nucleic acid, adenylic acid, and cozymase.\* The pyrimidines, uracil and thymine, were negative. Guanine was a strong inhibitor while guanosine was inactive. Adenine was weakly positive, while all adenine-containing physiological agents, adenylic acid, nucleic acid, and cozymase, were strongly positive. Of this series only adenylic acid was active at 0.1 mg. per 1 c.c. Table III lists results obtained with these agents.

TABLE II  
AMINO ACIDS INFLUENCING THE ANTISEPTIC ACTIVITY OF PROFLAVINE  
(Averages for four sets)  
(Values expressed as number of bacteria per c.c.)

AMINO ACID 0.5 MG./C.C.	PROFLAVINE + AMINO ACID 0.05 MG./C.C.	PROFLAVINE 0.05 MG./C.C.	CONTROL	AMINO ACID
Phenylalanine	200,000	47,000	189,000,000	37,000,000
Phenylalanine	16,800,000	150,000	25,800,000	
Tyrosine	1,404,000	2,539,000	63,000,000	
Tyrosine	3,000,000	150,000*	25,000,000	
Cysteine	5,670,000	520	94,800,000	
Cysteine	4,500,000	500	20,000,000	
Histidine	870,000	47,000	189,000,000	43,740,000
Histidine	750,000	500	20,000,000	
Glutamic	91,800,000	6,480	18,000,000	30,000,000

\*0.1 mg./c.c.

TABLE III  
THE EFFECT OF PURINES AND PURINE DERIVATIVES ON THE ANTISEPTIC ACTION OF PROFLAVINE  
(Averages for four sets)  
(Values expressed as number of bacteria per c.c.)

COMPOUND 0.5 MG./C.C.	PROFLAVINE + COMP. 0.05 MG./C.C.	PROFLAVINE 0.05 MG./C.C.	CONTROL	COMPOUND
Adenine	77,760	36	102,000,000	48,600,000
Adenine	56,160	120	25,000,000	24,000,000
Adenylic Acid	82,800,000	520	94,800,000	100,000,000
Adenylic Acid	27,600,000	56	36,000,000	
Cozymase	144,000,000	96	36,000,000	
Cozymase	3,250,000†	3,000,000*	30,000,000	
Nucleic Acid	5,400,000	120	25,000,000	30,000,000
Nucleic Acid	34,800,000	660†	70,000,000	
Guanine	232,000	47,000	189,000,000	7,290,000
Guanine	4,200,000	520	87,000,000	88,000,000
Guanosine	432	520	94,800,000	
Guanosine	1,070	540	87,000,000	

\*0.01 mg./c.c.

†0.08 mg./c.c.

‡0.1 mg./c.c.

Both adenine and guanine are positive, one is 6-aminopurine, the other 2-amino-6-oxypurine. Uric acid is negative. This may suggest that an amino group in the 2- or 6-position is vital to inhibitory activity. Thymine, 2:6-dioxy-5-methylpyrimidine, and uracil, 2:6-dioxypyrimidine, are both devoid of activity and of amino groups. However, the chemical reactivity of purines and pyrimidines is of such a similar nature as to render improbable chemical reaction between acridine and purines as the basis of the inhibitory action of these agents. Coenzyme 1, cozymase, is an adenine nucleotide containing in its molecule one

\*Obtained through the courtesy of Dr. E. Bueding and Dr. S. Ochoa of Bellevue Hospital, New York, N. Y.

mole of adenine, one of nicotinamide, and two of phosphoric acid and d-ribose. Adenylic acid is composed of one molecule each of adenine, ribose, and phosphoric acid. In each case, the amino group of the adenine moiety is free.

*Miscellaneous.*—p-Hydroxyphenylpyruvic acid (2.0 mg./c.c.) was found negative. This rather suggests that the activity of pyruvic acid is a specific one and not due to reaction of the carbonyl radical with the amino of proflavine. Blood serum was found highly potent as an inhibitor when added in quantities of 1 c.c. to the 10 c.c. of medium containing the proflavine. The activity seemed to be no greater than that found in 5 mg. of adenylic acid. Several of the specific inhibitors, e.g., pyruvic acid and malic acid, seemed to possess a potency in 5 mg. amount equal to that of 1 c.c. of serum. Casein hydrolysate tested at 100 mg. in 10 c.c. was active, and its activity could easily be accounted for by its content of glutamic acid, histidine, cysteine, phenylalanine, and tyrosine. Creatine and creatinine were tested and found negative.

Using a proflavine concentration of 1 in 1,000,000 at which it exerts a very weak antiseptic action, various sulfonamides were tested in concentrations of 1 in 100,000 for any inhibitory effect against proflavine. Sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, and sulfaguanidine were all negative. Pyridium was also devoid of inhibitory action under these conditions.

Many of the inhibitors of proflavine action were tested as possible stimulators of the growth of *Staphylococcus aureus* in the standard beef extract broth used as medium. Glucose, 10.0 mg./c.c., and phenylalanine, guanine, adenine, histidine, sodium oleate, adenylic acid, nucleic acid, pyruvic acid, acetic acid, succinic acid, and glutamic acid, each at 0.5 mg./c.c., were all tested, and of these only succinic acid and glutamic acid stimulated growth even to a slight extent. Details of results with the various agents are listed in Tables I, II, and III.

The ratio of proflavine to effective concentration of inhibitor was between 1:100 and 1:20. This ratio was maintained using concentrations of proflavine from 1:200,000 to 1:5000, where pyruvic acid and adenylic acid were studied as the inhibitors.

*Chemical Studies.*—Attempts were made to precipitate proflavine from a saturated solution by adding a saturated solution of adenylic acid, malic acid, pyruvic acid, and guanine. With the guanine and the malic acid a precipitate formed, but as was subsequently determined, this was due to a salting out process and not to the formation of an insoluble proflavine complex. At the concentrations employed in the bacteriologic testing, 1 in 200,000 for the antiseptic and 0.5 mg. per c.c. of the other reagents (unless they were less soluble), no precipitates were formed. It may easily be that soluble complexes are formed. In this connection, the work of Wagner-Jauregg<sup>17</sup> is interesting. He described the formation of various acridine salts of adenosinepolyphosphoric acids. These salts were formed with adenylic acid and with the adenosine di and tri phosphoric acid complexes. No precipitate occurred when cozymase was added to acridine solution. Under the conditions of our experiments, no precipitates were formed when proflavine and adenylic acid were allowed to react in saturated solutions. Our conditions attempted to simulate the bacteriologic experimentation conditions and did not resemble those of Wagner-Jauregg, who treated a watery solution of the free adenosinephosphoric acid with an alco-

holic solution of acridine. Thus, it is felt that although it is highly probable that complexes of some sort are formed between the adenine-containing compounds and proflavine, these complexes are not insoluble. More specifically, the basis of the mechanisms under consideration is not one of simple precipitation of the active growth-promoting physiologic ingredient of the medium. The fluorescence of a 1 in 200,000 solution of proflavine is not altered by the presence of 0.5 mg. per c.c. of either pyruvic acid or adenylic acid; this is further proof against any precipitation.

*Discussion.*—The action of the acridine antiseptics depends primarily upon the ability of these agents to inactivate physiologically important adenine compounds. In this group are adenine, adenylic acid, adenosine triphosphate, cozymase (coenzyme 1, which is a diphosphopyridine nucleotide), coenzyme 2 (triphosphopyridine nucleotide), and flavine adenine dinucleotide (xanthine oxidase, d-amino oxidase, diaphorase, aldehyde oxidase, and fumaric hydrogenase).

It is not impossible that part of the action depends upon the inhibition by the acridine of nucleosidase which is responsible for the liberation of adenine from nucleic acid combination. However, this seems unlikely because adenine itself is an extremely weak inhibitor of the action of the acridines, and if the presence of free adenine were the limiting factor one would expect it to be a powerful inhibitor.

When we consider each enzyme in its specific system along with the chemicals demonstrated to inhibit the antibacterial action of the acridines, it becomes apparent how they cross-check.

Fumaric hydrogenase is the yellow enzyme catalyzing the reduction of fumaric acid to succinic acid. Fumaric acid weakly inhibits the acridine activity; succinic acid powerfully inhibits this action. The fumaric acid probably acts by mass action, forcing some formation of succinic acid via the reduced effective concentration of the fumaric hydrogenase.

d-Amino acid oxidase but not l-amino acid oxidase is affected. As not all amino acids inhibit acridines, l-amino acid oxidase should not be involved. The fact that it is not may indicate that this enzyme is not an adenine-containing complex. Although d-amino acid oxidase is probably inhibited, the d-amino acids do not inhibit the acridines, since d-amino acid metabolism is probably not vital to the life processes of the *Staphylococci*.

Coenzyme 1 is demonstrated to inhibit the acridines. Its action is as a coenzyme for many dehydrogenases. During the reactions it takes up two hydrogen atoms, forming dihydrocoenzyme 1. It is the coenzyme for over 35 different enzymatic reactions. Glucose dehydrogenase converts glucose to gluconic acid, and both glucose and gluconic acid are weak inhibitors of proflavine.

Penicillin B<sup>3</sup> is an enzyme of the flavine type converting glucose to gluconic acid. It is possible that any enzymatic system may be the mechanism of antibacterial action if it is caused to become exceedingly active, producing an excess of an antibacterial chemical, or if it is caused to cease to act entirely, thus depriving the bacterium of a required nutrient material. Malic dehydrogenase converts malic acid to oxalacetic acid, and malic acid inhibits the acridines to a mild degree.

Lactic dehydrogenase converts lactic acid to pyruvic acid, and both acids inhibit acridines. The product, pyruvic acid, is by far the more powerful inhibitor. Glutamic acid dehydrogenase converts glutamic acid to alphaketoglutaric acid, and glutamic acid partially inhibits the acridines. These seem enough examples to demonstrate the point that coenzyme 1 is inhibited and that increased concentration of substrate or product aids the bacterium in overcoming the action of the antiseptic.

Adenylic acid is also an excellent inhibitor of acridines. This fact in turn indicates that the acridines inhibit adenylic acid. If this were true, it would lead to certain metabolic abnormalities which could be corrected by supplying phosphorylated forms or certain dephosphorylated forms. Thiamine is believed to be converted to cocarboxylase through the action of adenylic acid, and therefore cocarboxylase should partially inhibit the acridines. The fact that it does inhibit the acridines indicates the correctness of the deduction. Pyruvic acid is at one phase of intermediary carbohydrate metabolism the end product of the dephosphorylation of phosphopyruvic acid, which gives its phosphate radical to adenylic acid, which is in turn converted to adenosine triphosphate. Pyruvic acid is a powerful inhibitor of proflavine, and there would seem to be two reasons. First, it is the product of lactic acid dehydrogenase of which coenzyme 1 is an essential part, and second, it is one of the important products of intermediary carbohydrate metabolism at the point involving the adenylic acid molecule.

The manner in which phenylalanine and tyrosine fit into this picture is as yet undetermined. It is true that in certain instances bacteria have a metabolic requirement for one or the other. *Pasteurella pestis*<sup>6</sup> seems to require phenylalanine in preference to tyrosine for its growth. Neither tyrosine nor phenylalanine is required growth factor for *Bacillus larvae*.<sup>7</sup> Tyrosine alone is effective for *Streptococcus salivarius*.<sup>8</sup> The typhoid bacillus<sup>9</sup> seems to require neither tyrosine nor phenylalanine. In the case of the meningococcus<sup>10</sup> adequate growth is obtained with an amino acid mixture containing phenylalanine and no tyrosine. *Streptococcus hemolyticus*<sup>11</sup> requires seven amino acids, tyrosine, and not phenylalanine. *Bacterium tularensense*<sup>12</sup> grows well on a synthetic medium containing phenylalanine but no tyrosine. Thus, it cannot be definitely stated that *Staphylococcus aureus* requires phenylalanine or tyrosine for its metabolism, but the indications are that it does.

Our observations entirely confirm those of McIlwain<sup>3</sup> concerning the activity of phenylalanine and nucleic acid in inhibiting the antibacterial activity of proflavine. The general mechanism suggested by him we have confirmed and extended. The enzyme systems are the adenine-containing enzyme systems, and the substrates are not only certain amino acids but also various intermediary carbohydrate metabolic products.

Dickens<sup>13</sup> noted that acriflavine among other agents inhibited the Pasteur reaction, which might be stated to be carbohydrate oxidation under aerobic conditions. This is a mechanism in which the physiologically important adenine-containing compounds would be directly involved, and therefore Dickens' results fit into the picture as we construct it. It was felt by this investigator that the activity of the dyes was due to an anticatalytic action on certain cell enzymes, and he suggested that they might be pyridine-containing enzymes, or

coenzymes which were involved in hydrogen transport. He further suggested that the active agents displaced the coenzymes by preferential adsorption on the colloidal carrier, forming an inactive complex. This theory of Dickens is in essence the mechanism that we have established. Manifold<sup>14</sup> found that acriflavine, proflavine, and 2:7-diaminoacridine inhibited the pyruvate and glucose oxidation systems of brain tissue. The inhibition of fumarase by acriflavine was demonstrated by Quastel,<sup>15</sup> who later<sup>16</sup> demonstrated an inhibition of urease by this same chemical. In the urease experiments, Quastel found that glycine, p-aminobenzoic acid, aspartic acid, glutamic acid, and allantoin afforded nearly 50 per cent protection against brilliant green toxicity for urease.

All of the facts recorded by these investigators<sup>13-16</sup> fit into the theory we present for the mechanism of action of the acridine antiseptic, proflavine.

#### SUMMARY

Proflavine, 2:8-diaminoacridine, produces bacteriostasis in *Staphylococcus aureus* cultures by inhibiting adenine-containing physiologically important factors: adenylic acid, coenzymes 1 and 2 and adenine flavine dinucleotide. This fact was demonstrated by the inhibition of the action of proflavine by adenine, adenylic acid, cozymase, and nucleic acid (yeast). Further, metabolic intermediates of the action of these agents inhibit acridine; included among these intermediates are glutamic acid, lactic acid, acetic acid, pyruvic acid, and gluconic acid.

Certain agents which inhibit acridines and constitute exceptions to the above scheme are discussed.

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# CLINICAL CHEMISTRY

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## THE RELATIONSHIP BETWEEN BLOOD VOLUME AND BLOOD SPECIFIC GRAVITY IN THE RECOVERY FROM CARDIAC DECOMPENSATION\*

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AS a result of the work of Gibson and Evans,<sup>1</sup> it is now generally recognized that the recovery from cardiac decompensation is attended by a fall in blood volume which parallels improvement in venous pressure and circulation time. In severe instances of decompensation, this fall may be as much as 40 per cent of the initial volume. With such a marked reduction in blood volume over a relatively short period of time and also in consideration of the profuse diuresis in a compensating cardiac patient, the question naturally arises as to the effect upon the concentration of the blood. Does the blood become more concentrated, and, if so, does it affect the fluid portion of the blood as well as the cellular? Stewart<sup>2</sup> has presented evidence that the first period of recovery from decompensation is accompanied by a fall in plasma specific gravity which later rises to control levels as complete compensation is reached. Most authorities believe that there is a concentration of the blood, but only because the relative proportion of red blood cells to plasma increases as the patient in cardiac failure recovers.<sup>1, 3</sup> However, the exact relationship has never been worked out.

This investigation was undertaken in an attempt to answer these problems by frequent measurements of blood volume, whole blood specific gravity, plasma specific gravity, and hematocrit readings in a suitable group of patients with heart disease as they recovered from decompensation.

### METHODS AND PROCEDURES

Plasma volume was measured by the dye method of Hooper et al.<sup>4</sup> The method was modified only by substitution of the Evans' Blue, T 1824† for the dye vital red. Also, in recognition of the prolonged mixing time found in cardiac decompensation by Gibson and Evans,<sup>1</sup> a seven-minute period was allowed to elapse before collection of the second sample instead of four minutes. By use of graded standard solutions the unknown and standard readings

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†The dye Evans' Blue used in this study was obtained from the Chemical sales Division, Eastman Kodak Company, Rochester, N. Y.

on the colorimeter were never allowed to deviate more than 10 per cent. This greatly added to the accuracy of the results. From the plasma volume and the hematocrit reading, the whole blood volume and red cell volume were calculated.

Whole blood specific gravity and plasma specific gravity were measured by the method of Barbour and Hamilton<sup>3</sup> as perfected by Scudder.<sup>4</sup> Heparin was used as the anticoagulant. All samples were venous, secured from the cubital fossa without stasis. Rigid precautions were followed to prevent hemolysis, and all samples showing the slightest defect in this direction were discarded. Double determinations were made. Hematocrit readings were also performed in duplicate by the use of Sanford-Magath tubes. They were centrifuged at 2,500 revolutions per minute for one hour. Venous pressures were determined by the standard "L" tube method with the needle held 10 centimeters above the level of the bed. Circulation time from the arm to tongue was measured with decholin.

Only those patients with ample evidence of decompensation were selected for this study. They all had clinically obvious venous distention and peripheral edema. The procedure with any given case when first seen was as follows: A few hours after admission the initial blood volume, blood specific gravity, venous pressure, and circulation time studies were made. He was then rapidly digitalized by mouth. Fluid intake was limited to 1,200 c.c. daily. Approximately twenty-four hours later, an injection of 2 c.c. of mercupurin was given. This was repeated in four days when necessary. Each patient was then carefully followed for at least a two-week period. The blood volume determination as well as venous pressure and circulation time was repeated on the second or third day after admission and also on the fifth, ninth, and fourteenth days. Hematocrit readings and blood specific gravity studies were done daily. Blood samples for these latter determinations were always collected approximately three hours after a standard light breakfast.

#### RESULTS

Early in the investigation it was found in a large group of decompensated cardiac patients that if the specific gravity of the plasma was followed daily until compensation was attained, two types of curves resulted. In the first (Group I), the specific gravity of the plasma fell in the first two or three days following therapy and then rose gradually to control or just above control levels as compensation was achieved. In the second type (Group II), the specific gravity of the plasma stayed constant or rose in the first two or three days following the onset of recovery. The general character of these curves is illustrated in Figs. 1 and 2, respectively. Note that the specific gravity of the plasma has been converted to plasma protein by means of Weech's formula.<sup>5</sup> In order to elucidate further the nature of this change in blood concentration, sixteen careful experiments on fourteen patients were carried out; in these the specific gravity of the whole blood, hematocrit readings, and the blood volume were also studied. The age and type of heart disease were summarized in Table I. All were males with the exception of R. B. and A. B. Two patients

F.P. and B.T. were studied on separate admissions to the hospital. These were approximately three and two months apart respectively. Close study of Figs. 1 and 2 reveals additional factors of interest. In all cases except one, I.M., the change in hematocrit reading paralleled the change in plasma proteins; for example, if the plasma proteins fell, the red blood cell per cent also

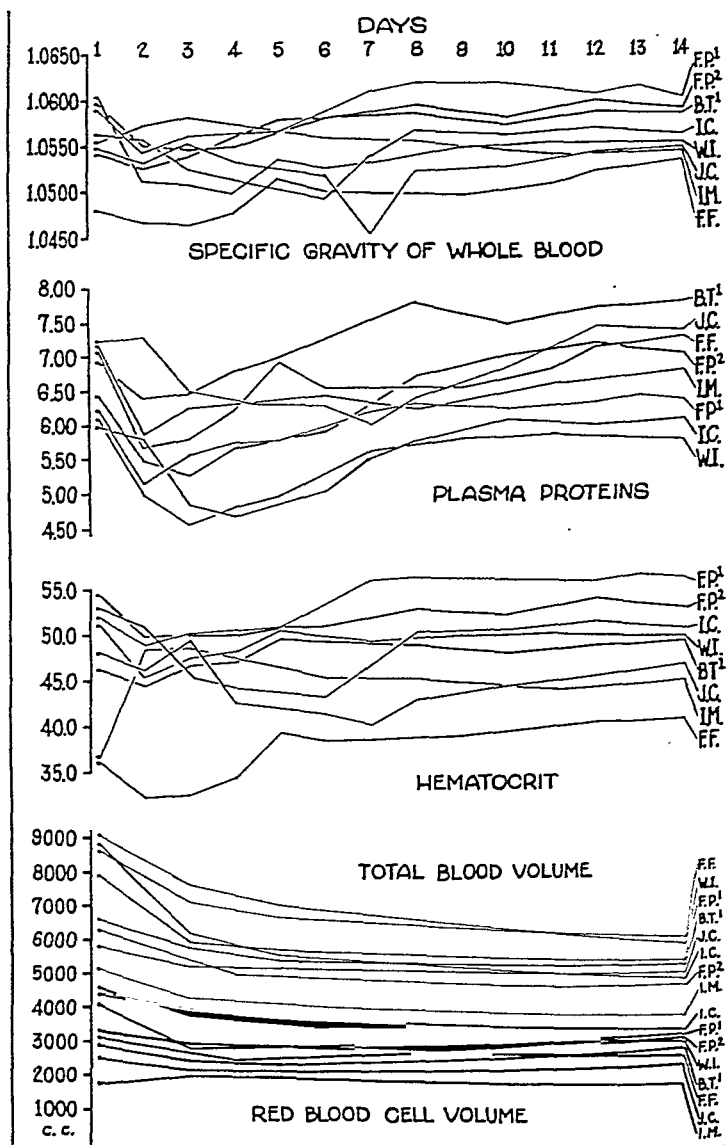


Fig. 1.

Figs. 1 and 2.—Group I and Group II cardiac patients are illustrated respectively (see Table I). The first day represents conditions before the onset of therapy, while the fourteenth day shows complete recovery from heart failure. The specific gravity of the plasma has been converted to plasma proteins by use of Weech's formula and has the same significance. Note that all the patients in Group I had a fall in plasma proteins during the first 2 or 3 days of recovery. The hematocrit reading also fell during this period in all instances except one. The whole blood specific gravity reflects the over-all change in blood concentration during recovery. Note that in the first period of recovery the plasma volume fell more rapidly than the red blood cell volume. Despite this apparent blood concentration, the whole blood specific gravity fell because of the simultaneous marked decrease in plasma proteins. Group II patients show either no change in blood specific gravity or a rise. This is associated with the lack of change in blood volume. (See text for full discussion.)



fell, and vice versa. This variation in plasma proteins and hematocrit readings was corroborated by the change in the specific gravity of whole blood. Thus in the first group, Fig. 1, the specific gravity of whole blood fell in every instance except in the case of I. M. who it should be noted had a rise in the hematocrit reading instead of a fall. In Fig. 2, the second group of patients also show a parallel variation of whole blood specific gravity, plasma proteins,

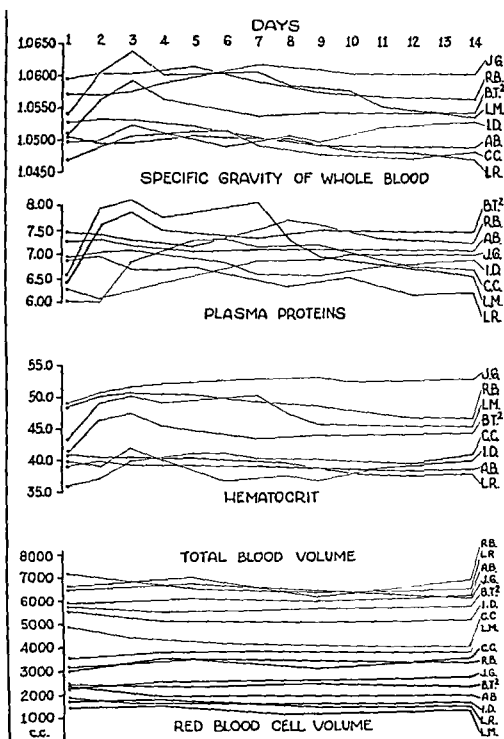


Fig. 2.

(See Fig. 1 for complete legend)

and the hematocrit. Thus the observed changes in whole blood specific gravity substantiate the conclusion that the deviation in the specific gravity of the plasma in one direction is attended by a change in the hematocrit reading in the same direction. The significance of this will be discussed below.

When the blood volume in each cardiac patient is studied in relation to the changes in blood concentration, one other fact stands out (Figs. 1 and 2, and

Table I). In the first group, the decompensated patients have a greatly increased initial blood volume, whereas in Group II the blood volume is not very much increased. Moreover, the decrease in total blood volume with compensation in the first group is marked, 28 per cent. In the second group, the decrease is only 6 per cent. This fall in blood volume is corroborated by the fact that the extent of the fall in venous pressure was also greater in the first group.

TABLE I

LIST OF GROUP I AND GROUP II CARDIAC PATIENTS WHICH ARE REFERRED TO IN THE TEXT.  
THE RELATIONSHIP BETWEEN THE DECREASE IN BLOOD VOLUME AND RECOVERY  
FROM HEART FAILURE AS INDICATED BY THE VENOUS PRESSURE AND  
CIRCULATION TIME IS DEMONSTRATED IN BOTH GROUPS

NAME	AGE	DIAGNOSIS	ON ADMISSION			RECOVERY		
			VENOUS PRESSURE CM. H <sub>2</sub> O	CIRCULA- TION TIME SEC.	TOTAL BLOOD VOLUME C.C.	VENOUS PRESSURE CM. H <sub>2</sub> O	CIRCULA- TION TIME SEC.	TOTAL BLOOD VOLUME C.C.
GROUP I								
F. P. <sup>1</sup>	50	H.C.V.D.	34.5	39	7915	11.0	13	5611
F. P. <sup>2</sup>	50	H.C.V.D.	29.0	46	6240	13.5	20	4895
B. T. <sup>1</sup>	51	L.H.D.	25.5	25	6521	9.0	15	5592
I. C.	70	H.C.V.D.	39.5	45	8717	15.0	35	4893
W. I.	56	A.H.D.	41.5	48	9061	13.5	24	5920
J. C.	58	H.C.V.D.	26.0	37	5722	16.0	19	5076
F. F.	64	L.H.D.	39.0	30	5124	10.5	17	3823
I. M.	66	A.H.D.	34.0	40	8734	19.0	20	6010
MEANS			33.6	38.7	7254	13.4	21	5227
GROUP II								
J. G.	51	A.H.D.	23.5	38	6620	10.0	9	6289
R. B.	50	R.H.D.	33.5	25	6646	29.0	28	6380
		H.C.V.D.						
B. T. <sup>2</sup>	51	L.H.D.	25.5	24	5846	15.5	10	5628
L. M.	86	A.H.D.	18.0	25	3598	12.5	12	3536
I. D.	72	A.H.D.	30.5	32	5602	11.5	19	5086
A. B.	48	T.H.D.	33.0	12	5941	22.5	9	6120
C. C.	74	A.D.H.	32.5	25	4960	19.0	14	3982
L. R.	58	H.C.V.D.	30.0	26	7210	15.0	17	6600
MEANS			28.3	25.9	5803	16.9	14.7	5452.6
A.H.D. Arteriosclerotic heart disease. H.C.V.D. Hypertensive cardiovascular disease. R.H.D. Rheumatic heart disease. T.H.D. Thyroid heart disease (thyroidtoxicosis). L.H.D. Leucic heart disease.								

Also of note is the fact that one cardiac patient studied, F. P., had the same type of curves on two separate admissions. His blood volume in both instances was initially elevated and was markedly reduced with recovery. In contrast, B. T. fell into the first group on his first admission and into the second group in his second attack of decompensation. The blood volume changes corresponded, for on his first admission the reduction in total blood volume with recovery was 929 c.c.; on his second admission it was only 218 c.c.

A correlation chart serves to illustrate graphically the relationship between the blood volume and plasma specific gravity in the two groups of cardiac patients (Fig. 3). In the first group it is seen that as the blood volume falls rapidly in the first three days of the onset of recovery from decompensation, the plasma protein concentration also falls rapidly. From the third day onward, while the blood volume is still diminishing but at a much slower rate, the plasma protein concentration rises gradually to slightly above its control level. In the second

group there is a slight increase in plasma protein concentration with the fall in total blood volume of the first three days. After this, there is little change in the relationship between the two factors. Since in the two groups the hematocrit readings and the whole blood specific gravity parallel the plasma proteins, it is obvious that the same relationship holds for the latter (see Figs. 1 and 2).

It may be concluded, therefore, that as a decompensated cardiac patient recovers, the specific gravity of the plasma may either rise or fall for a period of from two to three days following the onset of therapy. It then stabilizes at approximately control or slightly above control levels, with complete compensation. The hematocrit reading generally parallels the changes in plasma specific gravity. Those cardiac patients who have a fall in specific gravity of the blood have an increased initial blood volume which is markedly reduced with compensation, while those who do not have a fall in specific gravity of the blood do not have a large initial blood volume with marked reduction in the recovery phase.

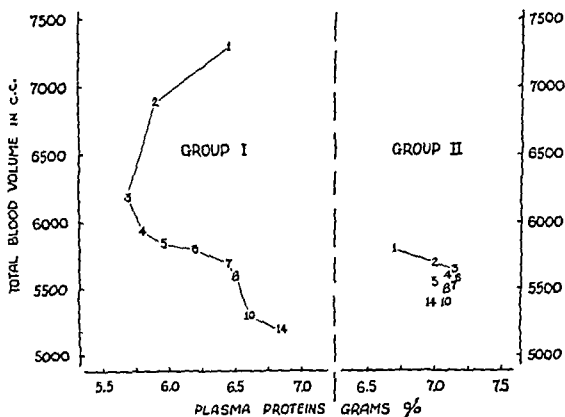


Fig. 3.—Correlation graphs of the changes observed in blood volume and plasma protein concentration. Data for graphs gathered from charts are shown in Figs. 1 and 2. Each numeral represents the mean total blood volume and the mean plasma protein concentration on each day that the patient was studied. Thus, numeral 1 represents the mean total blood volume and the mean plasma protein concentration on the first day before therapy was started. The succeeding numerals indicate the change observed on the following days until complete recovery takes place. Note that in Group I the rapid fall in blood volume in the first 3 days is attended by a corresponding rapid fall in plasma proteins. Thereafter, as the blood volume continues to diminish less rapidly, the plasma proteins rise gradually to their former level. In Group II the blood volume diminishes only slightly with recovery, and the plasma proteins in this instance rise slightly in the first three days and then remain constant.

#### DISCUSSION

In agreement with Gibson and Evans<sup>1</sup> it was found that during the initial period of recovery in the first group of patients (Fig. 1) the decrease in plasma volume was greater than the decrease in red cell volume. However, their supposition that because of this relative increase in red blood cell volume, the concentration of whole blood at this time must be greater is not borne out in fact.

They were not aware that during this period there is also a marked fall in the specific gravity of the plasma. The curves of whole blood specific gravity in Fig. 1 clearly show the fall in whole blood concentration during this period of recovery from decompensation. Stewart's<sup>2</sup> conclusions are also open to question in regard to increases or decreases in blood volume, as he studied changes in specific gravity of the plasma alone during recovery in cardiac patients. Therefore, in view of the evidence here presented it cannot be too strongly emphasized at this point that changes in blood concentration do not always indicate the impending changes in blood volume, nor, conversely do changes in blood volume always show the impending changes in blood concentration. *To determine accurately blood concentration or dilution, both specific gravity and blood volume studies must be made.* Disregard of this factor has led to much confusion in the literature, as aptly pointed out by Bazett.<sup>8</sup>

#### SUMMARY AND CONCLUSIONS

Sixteen experiments on fourteen patients with various kinds of heart disease during severe decompensation were made. As each patient recovered from decompensation with the usual therapeutic regime, the whole blood specific gravity, plasma specific gravity, and hematocrit readings were done daily. Blood volume was measured by the plasma dye method at least four separate times at suitable intervals during the recovery period. Venous pressure and circulation times were done frequently to gauge the extent of decompensation and recovery. The data secured permitted the following conclusions to be derived concerning the relationship between blood concentration and blood volume in the recovery from cardiac decompensation.

It was found that the decompensated cardiac patients studied could be separated into two groups. The first group had a high initial blood volume which was reduced markedly with recovery from decompensation. In the first two or three days following the onset of recovery there was a marked fall in specific gravity of the plasma. The hematocrit level also fell during this period in all cases except one. Later, with further recovery, there was a gradual rise in plasma specific gravity and hematocrit levels, to control or slightly above the control levels. The changes in whole blood specific gravity corroborated these findings.

The second group did not have a marked initial increase in blood volume and the reduction with recovery was slight. In this instance there was either little change in plasma specific gravity or a rise during the first period of recovery from decompensation. In all instances the hematocrit level paralleled the changes in plasma specific gravity.

In the light of these results, the significance of the relationship between blood concentration and blood volume, in patients' recovery from cardiac failure, was discussed. It was pointed out that to accurately determine blood concentration or dilution, both specific gravity and blood volume studies must be made.

The authors are grateful to Dr. J. Hamilton Crawford for his interest and helpful suggestions in this investigation.

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# LABORATORY METHODS

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## GENERAL

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### COMPARATIVE RESULTS WITH TRANSDERMAL (OR TRANSCUTANEOUS) AND INTRACUTANEOUS TUBERCULIN TESTS\*

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IT WAS pointed out previously<sup>1</sup> that the many divergent opinions on the value of the tuberculin test might be accounted for primarily by the degree of chemical and bacteriologic purity and stability of the tuberculin preparations, and the lack of a full understanding of the basis of the tuberculin reaction and those biologic factors which account for its fluctuations in the individual case. When properly evaluated in the light of any single biologic test, it is still classed among the best biologic tests known to medicine. In diagnosis or in surveys, a good evaluation of a properly performed tuberculin test can be of inestimable value; not exclusively in every case, to be sure, even though at times it may be the only available positive or negative evidence, but rather inclusively when all available findings are considered.

Now that it is understood that the tuberculin reaction, either local or general, is a quantitatively variable condition based not on tuberculosis *per se* but rather on the commonly and almost regularly associated condition, tuberculous allergy,<sup>2</sup> it is possible to evaluate the significance of this reaction and to avoid misinterpretation in man or animal, especially if general reactive ability is considered as one factor in the negative or positive phases of this reaction. It has been disclosed<sup>3</sup> experimentally that tuberculin (tuberculo-protein) anaphylaxis is not significant in the tuberculous, but specific tuberculo-immunity and specific tuberculo- (bacillary) allergy are of practical significance as two separate phenomena occurring, however, distinctly to variable extent in various phases of the disease. Although there appears to be no definite evidence of the liberation of tuberculin *in vivo* in tuberculosis as it occurs *in vitro*<sup>4</sup> in cultures of tubercle bacilli, there is a definite tuberculo-allergic hypersensitiveness to tuberculin, as distinguished from bacillary hypersensitiveness. Such tuberculo-allergic hypersensitiveness is developed during the tuberculous infection and responds by the familiar reaction when tuberculin (tuberculo-protein) is used for test by injection or suitably applied so adequate and intimate contact with

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the body tissues occurs. The quantitative aspects of this response are well known and evident from the past failure to accept any single quantity of tuberculin for test by injection.

In the early part of this century, the lack of pure tuberculins proved a decided handicap to scientific progress. Not until Long and Seibert<sup>5</sup> introduced methods of preparing chemically pure tuberculins was it possible to define the tuberculin reactions more accurately as to their significance or dosage. When chemically pure tuberculins were used by injection for diagnostic or survey purposes, the amount to obtain a positive reaction and still avoid too severe a reaction had to be definitely limited and defined. Graded tests became necessary because of the quantitative aspects of the tuberculin reaction in the individual case. However, such procedure proved much more satisfactory and accurate than the Pirquet scarification procedure, used almost universally in the early surveys of this century, in which a full strength tuberculin was applied to a scarified (Pirquet method) or punctured (Craig method) skin and resulted in a high percentage of positive reactions. It is well known now that full strength tuberculin can cause a fair percentage of false positive reactions when introduced into the injured or abraded skin. This was one of the original objections to the Moro Ointment test which required vigorous rubbing and consequent skin injury to attain the proper introduction of the tuberculin suspended in fats as an ointment.

To obviate either injection or scarification, Vollmer and Goldberger<sup>6</sup> introduced the patch test. This is essentially a dried (saturated) tuberculin on a piece of filter paper attached to adhesive tape, and it requires four days after application before the final reading. The main advantages claimed were that the test requires no instruments, can be performed by nurse or assistant, and eliminates danger of infection. The tuberculin is liquefied sufficiently by the natural moisture of the skin ("perspiration insensibilis"). However, the disadvantages to the patch test are the slow evolution of the reaction, four days compared with one to two days by injection, and the adhesive tape reactions occurring in certain individuals.<sup>7, 8</sup>

Recognizing the possibilities of the transdermal tuberculin test and using chemically pure tuberculin, one can point out<sup>1</sup> that the nature of the injected tuberculin reaction appears to limit the use of tuberculin for diagnostic purposes. A transdermal, or transeutaneous, tuberculin test with pure tuberculin would have the advantages of being based on rapid absorbability through intimate contact of a high concentration of tuberculin in soluble form, producing no general toxicity, and offering simplicity, speed, and visibility. Such a test might also be self-limited by the very positive reaction itself.<sup>9</sup> However, there were three features which precluded practical success, the complexity of preparation, the high cost of chemically prepared pure tuberculin, and the unsuccessful attempts to incorporate the ordinary tuberculins in a suitable adhesive to insure intimate contact with the skin without producing irritation or reactions of any kind in itself.

The primary purpose of obtaining a suitable tuberculin (tuberculo-protein) was achieved in the development of a highly potent autolytic (or plasmolytic) tuberculin,<sup>10</sup> bacteriologically and chemically pure. Its biologic activity was

proved identical to the tuberculin (tuberculo-protein) obtained in the natural filtrate from growing tubercle bacilli on a nonprotein synthetic medium. This tuberculin could be readily dried, pulverized, and incorporated into a transparent skin adhesive preparation, would dry within several minutes on the skin of man or animals, produced no tensive or irritation reactions, and possessed no antigenic properties when applied to the skin alone in either normal or tuberculo-allergic individuals.

To eliminate marked variation in the response to different commercial tuberculins,<sup>11</sup> all transdermal and intradermal tests to be reported used the same original bacillary cultures, and the autolytic tuberculin and a purified protein tuberculin were prepared from these to rule out variations and to make comparisons basically as scientific as possible. Preliminary experiments in guinea pigs revealed a striking parallelism of the negative and positive reactions obtained in normal, tuberculous, and tuberculo-allergic guinea pigs (prepared with viable avirulent or nonviable heat-killed tubercle bacilli suspended in mineral oil).

In order to do a satisfactory scientifically controlled test on well-studied cases from a diagnostic standpoint, intradermal and transdermal test materials were prepared. Colonel George F. Aycock, Chief of Medical Service of Fitzsimons General Hospital, presented the data on the individual cases studied, and these are summarized into the following compilation of results. The findings are presented with the intradermal (Mantoux) and the transdermal (autolytic) tuberculin tests in 103 cases studied at Fitzsimons General Hospital.

Of the 103 cases tested, 90 gave a positive transdermal test, which was read at 24 and 48 hours after application of the autolytic tuberculin. Eighty-nine of these 103 cases gave a positive intradermal reaction, graded in the usual fashion from 1 plus to 4 plus; 87 of these gave a positive response following the injection of 0.001 mg. of purified protein tuberculin, and two additional cases gave a positive response after giving 0.005 mg. of tuberculin. There was agreement in positive findings between the transdermal and intradermal tests in 80 cases (considering all grades of positive reactions to 0.001 mg.), while 82 agreed when the larger amount (0.005 mg. tuberculin) was given intracutaneously.

There were seven cases which gave a positive transdermal test and a negative intracutaneous test (all grades reaction to 0.001 mg.), and two of the latter were positive to the 0.005 mg. test. There were seven cases which gave a positive (all grades) intracutaneous test and a negative transdermal test.

There were 31 cases with a positive smear or culture for the presence of tubercle bacilli in the sputum, and all of these gave a positive intracutaneous and transdermal test except one of the latter. The patient in that case was critically ill at the time of test and displayed a decidedly retarded transdermal absorption.

Some of the details of the special cases of disagreement between the intradermal and transdermal tests are presented here. In evaluating a positive or negative reading medically, one can place no absolute value on either test when negative or when considered singly. The exception, as is so often true in medicine, warns us that a positive or negative finding alone without corroborative substantiating findings for such a diagnosis would not justify definite conclusions



in the individual case. It must also be remembered that all features of tuberculous allergy upon which the tuberculin test is based are not fully understood at the present time, although dosage of tuberculin and the general physical condition of the patient are acknowledged factors in a positive determination in the presence of a positive allergic condition.

CASE 1.—Critical case of tuberculosis; active, and advanced cavitation. The sputum smear was positive, the transdermal test was negative, and an intradermal test (0.001 mg. tuberculin) gave a 2+ reaction within forty-eight hours.

CASE 2.—Sero fibrinous pleurisy and multiple pulmonary cavities with a negative sputum smear, a 1+ intradermal test (0.001 mg. tuberculin), and a negative transdermal test.

CASE 3.—Multiple pulmonary calcifications; sputum smear negative for acid-fast bacilli; intradermal test (both 0.001 and 0.005 mg tuberculin) negative; and the transdermal test was positive.

CASE 4.—Asthmatic bronchitis and bronchiectasis with a negative sputum for acid-fast bacilli and negative (both to 0.001 and 0.005 mg. tuberculin) intradermal test; positive transdermal test.

CASE 5.—Left spontaneous hemopneumothorax, negative sputum smear for acid-fast bacilli, negative (both to 0.001 and 0.005 mg. tuberculin) intradermal test; positive transdermal test.

CASE 6.—Quiescent fibrous tuberculosis with a negative sputum smear, positive (3+ to 0.001 mg. tuberculin) intradermally, and a negative transdermal test.

CASE 7.—Bronchial asthma with a negative sputum, positive (1+ to 0.001 mg. tuberculin) intradermally; negative transdermal test.

CASE 8.—Chronic bronchitis with a negative sputum for acid-fast bacilli, negative (both to 0.001 and 0.005 mg. tuberculin) intradermally: positive transdermal test.

CASE 9.—Left lower lobe bronchiectasis with a negative sputum smear, negative (both 0.001 and 0.005 mg. tuberculin) intradermal test; positive transdermal test.

CASE 10.—Chronic bronchitis (supposed hemolytic *Streptococcus viridans*) with a negative sputum smear and a negative (0.001 and 0.005 mg tuberculin) intradermal test; positive transdermal test.

CASE 11.—Pulmonary fibrosis with a negative smear, negative concentration test, positive culture for tubercle bacilli, a negative (0.001 and 0.005 mg tuberculin) intradermal test, and a positive transdermal test.

CASE 12.—Active tuberculosis with cavitation and a negative sputum smear, a positive (2+ with 0.001 mg. tuberculin) intradermal test, and a negative transdermal test.

CASE 13.—Active tuberculosis III C with a positive smear and positive intradermal test (2+ with 0.001 mg. tuberculin); a negative transdermal test.

CASE 14.—Catarrhal bronchitis and bronchiectasis with a negative smear and negative intradermal test; a positive transdermal test.

The foregoing disagreement in findings brings out several important points with regard to tuberculin tests. Even though highly specific, (1) these tuberculin tests are conditioned by a number of important factors not always controllable no matter which type of test is used; and (2) the importance of repeating a test easily if necessity demands. For this purpose, the transdermal test possesses many advantages over any injection test. However, it must be admitted that an intradermal test is warranted as a verification test at times, but the transdermal test appears to be preferable as a routine either for diagnosis or surveys.

Since it is duly recognized that no single biological test is infallible, a comparison of the transdermal and intradermal tests was made on the basis of

tuberculous and nontuberculous cases (including tuberculous infection). There were 64 of the former and 39 of the latter. The transdermal tests were read only as positive, indicating a definite reaction, or negative. Gradings can be made, but they do not seem essential with the transdermal test because of the self-limited nature of the reaction. In comparison, the intradermal tests may range from questionable 1 pluses to violent and extensive 4 plus reactions. Even in the tuberculous, the transdermal reactions never assumed alarming proportions.

Among the 64 tuberculous cases, 58 had a positive transdermal test and 62 had a positive intradermal test. Nineteen of these 62 reactions were on the borderline 1+ reaction, 31 gave a 2+ reaction, 11 a 3+ reaction, and 1 a 4+ reaction. If we include all reactions from 1+ to 4+, only four more positive cases were noted with the intradermal than with the transdermal test. Since the advanced condition in some of these cases would place them in the retarded absorption class not requiring a tuberculin test for diagnosis, it is obvious the transdermal test closely approximates the intradermal test in the definitely tuberculous.

Among the 39 nontuberculous cases (including those with possible tuberculous infection but no easily discernible disease), the transdermal test was positive in 32, while the intradermal test was positive in 25 with the 0.001 mg. tuberculin, and in 27 with both the 0.001 and 0.005 mg. tuberculin. The graded reactions for the intradermal tests were ten 1+ (1 with 0.005 mg. tuberculin), twelve 2+, and five 3+ (one with 0.005 mg. tuberculin).

Again the comparisons of the positive reactions were fairly close for the two tests. Since the reaction of the transdermal test depends upon the same specific tuberculin or tuberculo-protein and since none of the other constituents of the preparation react in any way in either nontuberculous or tuberculous patients, it must be assumed that any positive reaction to the specific tuberculo-protein tuberculin is caused by tuberculous infection or the presence of tuberculous (tuberculin) allergy in the sense that tuberculin elicits a response in the allergic hypersensitive organism.

#### SUMMARY

Comparison of the results of a transdermal tuberculin test (prepared from an autolytic tuberculin) and an intradermal test with a purified protein tuberculin (prepared from the same original bacillary cultures) on 103 patients revealed a striking parallelism between the two tests.

Among the 103 patients tested, 90 (87 per cent) were positive by the transdermal test and 89 (86 per cent) by intradermal injection. Agreement between the two tests occurred in 82 of the positive tests. When considering the 64 definitely tuberculous cases tested, the agreement was 58 (90 per cent) for the transdermal and 62 (96 per cent) for the intradermal tests. The slight differences were attributable to the transdermal absorption retardation in the definitely ill cases. Among the 39 nontuberculous cases (including those with possible infection but no evident tuberculous disease), the agreement again was fairly close; 32 (82 per cent) reacted to the transdermal test as compared with 27 (70 per cent) for the intradermal test.

It may be well to repeat here that the tuberculin test ranks among the best biologic diagnostic tests, not as an exclusive test but rather as an inclusive one to other verifying medical findings. It is valuable in specific surveys and as a diagnostic aid in tuberculosis and for the presence of the hypersensitivity of tuberculous allergy.

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# A MODIFIED TEST FOR THE DETECTION OF NITRATE REDUCTION\*

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FOR the past four decades the ability of bacteria to reduce nitrate has been an important characteristic in the identification of species. That the literature as yet contains contradictory findings has been indicated by Tittsler<sup>1</sup> in two members of the *Salmonella* group, Zobell<sup>2</sup> in the *Brucella* group, and Reed<sup>3</sup> in the clostridia group.

Conn,<sup>4</sup> in an evaluation of the nitrate reduction, has pointed out that simple tests which give a positive reaction indicate that reduction has occurred, but that to show that reduction does not occur is a complicated undertaking. In a test which is negative for nitrite and shows no visible gas production, there occur a number of explanations. The nitrate may be rapidly reduced to nitrite (with no accumulation) and further reduced to ammonia; the ammonia may be reduced to nitrogen; in insufficient quantity to be detected; the nitrite or ammonia produced may be assimilated; there may be a temporary loss of the organism to reduce nitrate; or the medium may not be suited to the purpose of the test. Zobell<sup>2</sup> has shown that, in a negative nitrite test, the addition of zinc dust will reduce the remaining nitrate to nitrite; a repeated negative test indicating that nitrate has been utilized and probably reduced, without nitrite accumulating.

This paper concerns itself primarily with the detection of nitrite as evidence of bacterial reduction of nitrate and will attempt to show, by using a modification of Bratton and Marshall's<sup>5</sup> technique for blood sulfonamide levels, a test of greater sensitivity and easier readability than those previously described by Wallace and Neave<sup>6</sup> and Tittsler.<sup>1</sup>

The investigation was divided into two parts: (1) evaluation of the relative sensitivities of the modified and sulfanilic acid tests by chemical means, and (2) evaluation of sensitivities and earliest detectability by microbial means.

## REAGENTS AND CULTURE MEDIA

### *Sulfanilic Acid Test Reagent:*

Solution (a) Sulfanilic acid	8 Gm.
Dilute sulfuric acid (5 per cent by volume)	1,000 c.c.
Solution (b) Dimethyl-alpha-naphthylamine*	6 c.c.
Dilute acetic acid (30 per cent)	1,000 c.c.

To each test was added 0.5 c.c. of (a) followed by 0.5 c.c. of (b).

Readings were made at the end of fifteen minutes.

These solutions keep for apparently not more than one month at room temperature.

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*Modified Test Reagent:*

Solution (a) Sulfanilamide* (saturated aqueous solution)	20 c.c.
Trichloroacetic acid (15 per cent)	80 c.c.
Solution (b) N-(1-naphthyl) ethylenediamine*	
dihydrochloride	0.1 Gm.
Distilled water	100 c.c.

To each test was added 0.5 c.c. of (a) followed by 0.5 c.c. of (b).

Readings were made immediately.

These solutions apparently keep indefinitely, at refrigerator temperature.

*Dunham's Peptone Broth:*

Peptone (Difco)	10 Gm.
Sodium chloride	5 Gm.
Distilled water	1,000 c.c.
No pH adjustment.	

The medium was tubed in 6.0 c.c. amounts, autoclaved at 121° C. for twenty minutes. A sterile (Seitz-filtered) 1.2 per cent aqueous solution of potassium nitrate (chemically pure nitrite-free) was added aseptically, 0.1 c.c. per tube, giving a concentration of 0.02 per cent potassium nitrate.

*Tittsler's Peptone Broth:*

Peptone (Difco)	2 Gm.
Potassium nitrate (chemically pure nitrite-free)	0.2 Gm.
Distilled water	1,000 c.c.
No pH adjustment.	

The medium was tubed and autoclaved as above, giving a concentration of 0.02 per cent potassium nitrate.

## EXPERIMENTAL

(1) To evaluate the relative sensitivity of the tests and substances interfering with it, a freshly prepared 0.1 per cent aqueous solution of sodium nitrite was prepared. Distilled water, 0.85 per cent sodium chloride, and the peptone broths previously described were used for subsequent dilutions. These dilutions, prepared in duplicate, were tested for the presence of nitrite by the addition of the modified and sulfanilic acid reagents, using 1.0 c.c., 0.5 c.c., and three drops of each solution.

Maximum color development occurred immediately on the addition of solution (b) of the modified test, but required fifteen minutes standing after the addition of solution (b) of the sulfanilic acid test. The observed sensitivities, using a readily discernible end point (1+) and all three amounts of reagents, were: in the modified test, 1 part nitrite in 100,000 parts water; in the sulfanilic acid test, 1 part nitrite in 1000 parts water; or an apparent one hundredfold sensitivity of the modified over the sulfanilic acid test.

\*Dimethyl- $\alpha$ -naphthylamine from Eastman Kodak Co., Rochester, N. Y. N-(1-naphthyl) ethylenediamine dihydrochloride from LaMotte Chemical Products Co., Baltimore, Md.

Sulfanilamide was selected because of its relative cheapness.

Dilutions prepared in 0.85 per cent NaCl solution gave essentially the same results.

Dilutions were then prepared, using as diluents the culture media described and the test reagents added as before.

TABLE I

CONC. OF NaNO <sub>2</sub>	DILUTION PREPARED IN DISTILLED WATER					
	MODIFIED			SULFANILIC ACID		
	1 c.c.	0.5 c.c.	3 drops	1 c.c.	0.5 c.c.	3 drops
1:100	4+	4+	4+	3+	3+	3+
1:1,000	4+	4+	4+	1+	1+	1+
1:10,000	3+	3+	3+	0	0	0
1:100,000	1+	1+	1+	0	0	0
1:1,000,000	±	±	±	0	0	0
1:10,000,000	0	0	0	0	0	0

4+ = maximum color developed (deep purplish red).

$\left. \begin{matrix} 3+ \\ 2+ \\ 1+ \\ \pm \end{matrix} \right\} = \text{varying degrees of color density, from a 4+ through a faint, although discernible, pink } (\pm).$

Here, it will be observed (Table II), the presence of peptone appreciably reduced the sensitivity of both tests. Using the same end point as in Table I it will be noted that the modified test gave only a 1:10,000 sensitivity, using Dunham's broth and 0.5 c.c. of each solution, as compared to a 1:1000 sensitivity with the sulfanilic acid test. This is perhaps not so striking in the dilutions with Tittsler's broth, which contains only one-fifth the amount of peptone. It was also noted that in using three drops of solutions (a) and (b) of the modified test, a deep reddish amber color was produced, entirely different in shade from all other colors developed. For this reason, the amounts of each test solution to be used in subsequent experiments was arbitrarily set at 0.5 c.c.

(2) To evaluate the relative sensitivities of the two tests by microbic means the following procedures were carried out:

Stock cultures of nine known positive and negative nitrate-reducing organisms were selected from the stock culture collection, plated for purity, and transferred on nutrient agar slants for three successive days. These growths were then suspended in 0.85 per cent NaCl solution, their densities adjusted to a BaSO<sub>4</sub> standard No. 3, and 0.1 c.c. of each suspension was added as an inoculum to a series of tubes containing Dunham's broth + KNO<sub>3</sub> and Tittsler's broth.

These were then incubated at 37° C. and tested for the presence of nitrite by the addition of 0.5 c.c. of each of the solutions previously described, at intervals of 4, 8, 12, 24, 48, and 72 hours after inoculation.

It will be noted (Table III) that although Dunham's broth + KNO<sub>3</sub> gave a more rapid and luxuriant growth (probably because of the increased concentration of peptone), this factor did not appreciably enhance the early detection of nitrite with either test. The nitrite-positive organisms rapidly reduced the nitrate present, and, in one instance (*Escherichia coli*), produced a confusing color reaction.

In our opinion, Tittsler's broth was the superior medium. It readily supported growth of all the test organisms used; a clear and maximum color production with both tests was noted in the usual period of incubation (12 to 24 hours), and confusing color reactions were absent.



TABLE III

TEST ORGANISM	TEST USING DUNHAM'S BROTH + KNO <sub>3</sub>											
	MODIFIED						SULFANILIC ACID					
	4 HR.	8 HR.	12 HR.	24 HR.	48 HR.	72 HR.	4 HR.	8 HR.	12 HR.	24 HR.	48 HR.	72 HR.
<i>E. coli</i>	4+*	4+*	3+	0	0	0	1+	±*	±	0	0	0
<i>P. vulgaris</i>	4+	4+	4+	3+	0	0	2+	2+	2+	2+	0	0
<i>E. typhosa</i>	4+	4+	4+	3+	0	0	2+	2+	2+	1+	0	0
<i>S. choleraesuis</i>	4+	4+	3+	3+	0	0	3+	2+	2+	2+	0	0
<i>Staph. aureus</i>	4+	4+	3+	3+	0	0	2+	2+	1+	1+	0	0
<i>Strep. fecalis</i>	0	0	0	0	0	0	0	0	0	±	0	0
<i>G. tetragena</i>	0	0	4+	±	0	0	0	0	1+	0	0	0
<i>P. aeruginosa</i>	4+g	±	0	0	0	0	1+g	0	0	0	0	0
<i>K. pneumoniae</i>	4+	4+	4+	4+	4+	4+	1+	2+	2+	2+	1+	1+
Blank	0	0	0	0	0	0	0	0	0	0	0	0
TEST ORGANISM	TESTS USING TITTSIEB'S BROTH											
	MODIFIED						SULFANILIC ACID					
	4 HR.	8 HR.	12 HR.	24 HR.	48 HR.	72 HR.	4 HR.	8 HR.	12 HR.	24 HR.	48 HR.	72 HR.
<i>E. coli</i>	4+	4+	4+	4+	4+	4+	1+	2+	1+	2+	2+	2+
<i>P. vulgaris</i>	4+	4+	4+	4+	4+	4+	1+	2+	3+	2+	2+	2+
<i>E. typhosa</i>	4+	4+	4+	4+	4+	4+	±	1+	2+	2+	2+	2+
<i>S. choleraesuis</i>	4+	4+	4+	4+	4+	4+	1+	1+	2+	2+	2+	2+
<i>Staph. aureus</i>	4+	4+	4+	4+	4+	0	1+	2+	2+	2+	2+	2+
<i>Strep. fecalis</i>	0	0	0	0	0	0	0	0	0	0	0	0
<i>G. tetragena</i>	0	0	3+	4+	4+	4+	0	0	0	2+	2+	2+
<i>P. aeruginosa</i>	4+g	0	0	0	0	0	1+g	0	0	0	0	0
<i>K. pneumoniae</i>	4+	4+	4+	4+	4+	4+	1+	2+	2+	2+	2+	2+
Blank	0	0	0	0	±	0	0	0	0	0	0	0

\*Color is reddish-amber.

g = Gas bubbles.



As a whole, the modified test gave an average increase of two-plus in color density over the sulfanilic acid test.

#### SUMMARY

A modified test for the detection of nitrite as an indicator of bacterial reduction of nitrate was described, based on the color produced by sulfanilamide and coupling reagent in the presence of nitrite.

The modified and sulfanilic acid tests were compared in relation to their sensitivity, ease of reading, and effect of interfering substances.

The nitrate-reducing ability of a group of pathogenic bacteria was evaluated, using Dunham's and Tittsler's peptone broths and the modified and sulfanilic acid tests.

It is believed that the nitrite test in present use is capable of improvement, and the modified test is presented here as a suggested step in that direction.

While the investigation was limited to human pathogens that grew well in liquid peptone media, there is little doubt that the modified test could be applied as well to saprophytes and plant parasites which grow poorly in such media.

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## A SIMPLE ELECTRON TUBE DROP RECORDER\*

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THERE are a number of drop recorders on the market, and several others have been described in the literature or at various meetings. These recorders are expensive, difficult to adjust, and are frequently unreliable.

The recorder shown in the diagrams is inexpensive, easy to use, operates on ordinary lighting circuits, is not affected by radiant heat, does not require suction, and has only one moving part. In extensive use it has given satisfactory service over many hours without attention. As drawn, it will record drops of any solution having as good conductivity as distilled water at any rate of drop formation up to fusion of the drops into a steady stream; at this rate it necessarily fails.

The wiring diagram for the essential element, a radio receiving tube (No. 117N7GT), is shown in Fig. 1. For solutions of poor conductivity the bias of six megohms is necessary; for physiologic secretions the bias may be as low as 0.5 to 1.0 megohms. The condenser is an ordinary electrolytic one of 16 microfarads capacity. The power source is the usual 110-120 volt lighting circuit, either alternating or direct current. On direct current, polarity is important: if the tube fails with the plug in one position, reversal of the plug is necessary; no damage is caused by the failure to plug in correctly.

The insert in the first figure shows the simplest type of dropper to construct; simplicity of construction is gained at the expense of convenience in use. In Fig. 2 is shown a far more convenient dropper which is a trifle more difficult to construct, but which repays in convenience many times over. The current flowing through the dropper, with good metallic contact, is in the order of not over two microamperes; when the circuit is through a solution across the contacts, the higher resistance still further reduces the current. With such negligible current, electrolysis of the dropper parts is absent and base metals are used in their construction. We have found that any hypodermic needle of the luer slip type makes a good dropper. The needle is first bent at 90° by inserting the cleaning stylus, then by heating and bending. By having several gauges of needles available, the drop size can be easily regulated.

The actual record of the drops is traced on a kymograph by a writing tip cemented onto the armature of a telephone line relay,† which acts here as a signal magnet. The armature of the relay is the only moving part of the drop recorder.

\*From the University of North Dakota, Departments of Physiology and Pharmacology, and Electrical Engineering.

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†Western Electric Company, code number E-165.

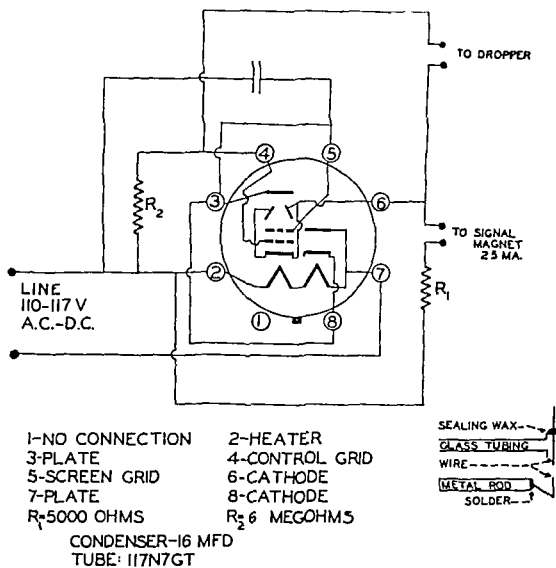


Fig. 1.—Wiring diagram for drop recorder.

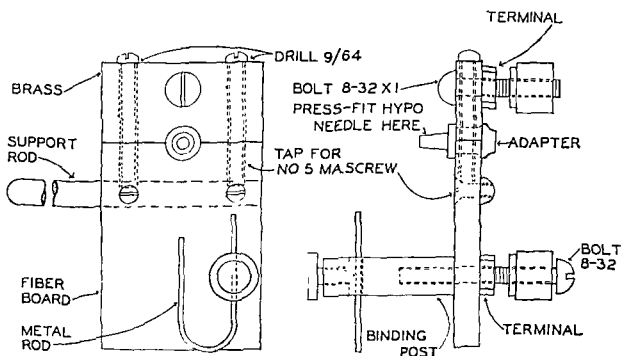


Fig. 2.—Dropper for drop recorder.

In use, the desired duct is cannulated. To the cannula one end of a rubber tube is attached; the other end is slipped over a hose-to-luer slip needle adapter.\* The adapter is then slipped into an identical adapter permanently mounted in the dropper. Onto the other end of the mounted adapter is slipped the bent hypodermic needle. Air in the rubber tube is displaced through the assembly by injecting saline solution into the tubing with a syringe, the needle being inserted into the lumen of the tube as near the cannula as possible.

The only failure of the apparatus occurred while recording the flow of very viscous bile: a thread of bile remained spanning the gap of the dropper, completing the circuit and holding the armature down. This condition is easily remedied by increasing the distance between the metal rod and the needle, or, if desired, a U-tube of saline solution may be inserted behind the dropper, with the secretion displacing the saline solution through the dropper.

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\*Becton, Dickinson, and Co., No. H/L/L.

## METHOD TO DETERMINE THE PENETRATING POWER OF GERMICIDES\*

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IT HAS been pointed out repeatedly that a germicide† must have the power to penetrate different kinds of substances (living tissues, mucus, many kinds of inanimate material) in order to get in contact with the germs to be killed, since these very often are embedded in such substances, and accordingly, are more or less protected against the killing power of the germicide.<sup>1-6</sup> This fact has been expressed lately by F. C. Brown and E. E. Reid: "Obviously sterilization cannot extend beyond the zone of penetration."<sup>7</sup>

The interposition of a layer which separates the germicide from the micro-organisms inhibits the killing power in several ways: (a) The separating substance alters the germicide chemically; (b) it adsorbs the germicide physically (Effront<sup>8</sup>). These possibilities may occur singly or combined. Their action diverts the germicide from its object, the germs to be killed. This effect could be designated as deviation. (c) The necessity to penetrate the separating layer requires time. The length of this period is influenced by the material of the separating layer, its thickness, the kind of the germicide, temperature, etc.

Although the necessity of testing the penetrating power of germicides has been acknowledged, only few attempts have been made to obtain experimental data.

Lubenau<sup>9</sup> in 1907 used little hornsticks, 2 to 3 mm. in diameter, which were allowed to imbibe a bacterial suspension. After drying, the infected hornsticks were tested against different disinfectants. In 1929 Lubenau again published his method.<sup>10</sup> The use of horn as a biologic material seems to be justifiable. However, the method gives no disclosure about the distance penetrated by the disinfectant in a given period.

A method for determining the germicidal value and the penetrating power of liquid disinfectants was established by Kendall and Edwards.<sup>11</sup> Coli-infected agar cylinders, 15 mm. in diameter, were submerged in the disinfectant, then washed with sterile water. From the center of the cylinder a core of 3 mm. diameter was removed and transferred into lactose fermentation tubes. This method is not only complicated, but introduces several sources of error (von Gutfeld and Gurwitz<sup>12</sup>). Furthermore, the procedure can be used for a limited variety of germs only.

The well-known cup-plate method by L. C. Himebaugh, as described in circular No. 198 of the U. S. Department of Agriculture, seems to solve the

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†The expression germicide comprises fungicidal, bactericidal, and viricidal agents.

problem of measuring the penetration of different kinds of germicides.<sup>13</sup> However, the "sterile" zone around the cup must be tested, whether the emptiness of this area is due to a bactericidal or a bacteriostatic action: "the agar in the clear zone may be tested for growth by subculture in broth."<sup>13</sup> The necessary transfer of small pieces of agar, cut from various sites of the sterile zone, involves sources of error and is rather cumbersome. Besides, the following criticism may be mentioned.

In the cup-plate method the clear zone around the cup is supposed to indicate how far the chemical penetrated the nutrient medium in the horizontal direction. If, for example, the cup is of 15 mm. diameter and the clear area 33 mm. in diameter, the conclusion would be that the chemical was still active after penetrating 9 mm. of the nutrient medium. This conclusion is not necessarily correct. It could happen that the active substance "migrated" by capillarity over the surface of the nutrient medium for that distance, and penetrated only the thickness of the nutrient medium from the surface downward. If 15 c.c. of the nutrient medium had been poured into the Petri dish of 9 cm. diameter, the agar layer would be ca. 2.4 mm. thick. This would mean that the penetrated stratum was not 9 mm., but 2.4 mm. only.

In the beginning of the penetration experiments\* the attempt was made to apply a "coating" to infected dried cotton strings. This technique proved to be of no avail since it was impossible to produce a flawless coating on the necessary number of strings. Finally the following technique was found satisfactory: Cotton strings, ca. 15 mm. long, ca. 1 mm. thick, were sterilized, infected, and dried. Ten c.c. of agar were poured into a sterile Petri dish. After the agar had hardened, the infected strings were laid on the surface of the agar, and 10 to 15 c.c. of agar (45° C.) poured over the strings. The strings now were embedded. After hardening of the covering layer, the germicide was poured on top of it. After varied periods of time a string was removed, thoroughly rinsed with sterile water, and transferred to a suitable nutrient medium.

It may be pointed out that the description of the method given above constitutes only an outline. Cotton strings with dried adherent germs are an example for the carrier. Instead of cotton strings any other suitable carrier may be used.<sup>14</sup> The germs may be fungi, bacteria with or without spores, or virus; either in pure cultures or in combinations. The embedding substance allows variations of the material (agar, gelatin, etc., with or without additions), of its concentration, and of the thickness of the covering layer. Different germicides may be used in various amounts and concentrations. Finally the temperature at which the test is performed can be varied.

The method may tentatively be named technique of "embedded germ carriers." It simulates natural conditions insofar as the germicide has to penetrate a covering layer whereby deviation may or may not take place.

*Experiment 1.*—Sterilized cotton strings were infected with a suspension of *Escherichia coli* and dried in the incubator. Ten c.c. of sterile 3 per cent gelatin is poured into a sterile Petri dish. After the gelatin has solidified in the refrigerator, coli-infected strings are laid on the surface of the gelatin.

\*The first experiments were performed with the assistance of Mr. L. G. May.

Of the same 3 per cent gelatin, 15 c.c. are poured over the strings, whereby a covering layer of about 2.4 mm. is produced. The Petri dish with the embedded strings and another Petri dish containing coli-infected strings only (in the following tables referred to as "direct") are placed in the refrigerator (temperature ca. +10° C.). After solidification of the covering layer of gelatin, a solution of phenol 1:100 in tap water is poured over the covering gelatin as well as over the strings in the other Petri dish. After varied periods of time one string from each series is removed, rinsed with sterile water, transferred to a test tube containing 5 c.c. of 1 per cent peptone water, and incubated at 37° C. The viability of the bacteria on dried control strings was tested before the start and after the end of the experiment: Both tests showed that the bacteria were alive and able to multiply.\* The results of this experiment are given in Table I.

TABLE I

[illegible]

The experiment demonstrates that it took more than 15 days for the penetration of the 1 per cent phenol solution through a layer of 3 per cent gelatin ca. 2.4 mm. thick at a temperature of ca. +10° C. By direct contact the same phenol solution killed the bacteria after more than 4, but less than 23 hours.

The same day another "direct" experiment was carried out at room temperature (ca. 28° C.). Here the colon bacilli were killed within 25 minutes.

The influence of the temperature on the disinfecting process is well known, and the slowing effect of the gelatin layer is not surprising. This retarding action may be caused by two different actions: the deviation of the germicide and the necessity to penetrate the gelatin.

The deviating action of gelatin on phenol could be demonstrated by the following experiments.

*Experiment 2.*—Coli-infected, dried cotton strings were exposed to the action of 1 per cent phenol in presence and in absence of gelatin respectively.

Over infected strings in separate Petri dishes were poured:

- (a) 18 c.c. phenol (1:90) + 2 c.c. 3 per cent gelatin, and  
(b) 20 c.c. phenol (1:100).

The experiment was carried out in the refrigerator (ca. +10° C.). Under these conditions the 1 per cent phenol killed the colon bacilli within 10 hours; addition of 0.3 per cent gelatin prolonged the killing time up to more than 23 (less than 26) hours.

*Experiment 3.*—Coli-infected dried cotton strings were treated with:

- (a) 8 c.c. phenol (2 per cent) + 8 c.c. 6 per cent gelatin = 1 per cent phenol, 3 per cent gelatin, and

\*These controls were performed in all our experiments and will be mentioned as "control start" and "control end."

(b) 8 c.c. phenol (2 per cent) + 8 c.c. sterile water = 1 per cent phenol.

Immediately after (a) and (b) have been prepared, the two liquids are poured over the strings in separate Petri dishes. The experiment was carried out in duplicate at room temperature (+27° C.). The results are given in Table II.

The results of the duplicated experiment were identical; all results were confirmed by subculturing.

The presence of gelatin increased the time necessary to kill the bacteria considerably, and so demonstrates its "deviating" effect.

The influence of the concentration of the disinfectant was tested in the following experiment.

*Experiment 4.*—Coli-infected cotton strings were embedded in 1 per cent agar; the covering layer is ca. 2.4 mm. thick. The technique of embedding has been described in Experiment 1. One batch of embedded strings is treated with 1 per cent phenol, another batch with 2 per cent phenol. The results of this experiment, which was performed at room temperature (28° C.) are given in Table III.

TABLE II

	MINUTES						
	2	5	10	20	30	45	60 90
1 per cent phenol + 3 per cent gelatin	+	+	+	+	+	+	- -
1 per cent phenol	+	+	-	-	-	-	- -
Control start: + Control end: +							

TABLE III

	COLI NOT YET KILLED AFTER	COLI KILLED AFTER
1 per cent phenol	55 hours	60 hours
2 per cent phenol	4 hours	8 hours
Control start: + Control end: +		

The increase in concentration shortens the time necessary to penetrate the covering layer and to kill the germs noticeably.

It seemed worthwhile to examine the effect of substances added to the gel on the penetrability of the mixture.

*Experiment 5.*—Coli-infected cotton strings were embedded in:

(a) 3 per cent gelatin, and

(b) 3 per cent gelatin in which 0.2 per cent lecithin ("practical," Eastman Kodak) was dissolved. The covering layer was ca. 2.4 mm. thick, the germicide was 1 per cent phenol, the temperature +10° C. At the same time infected strings were directly treated with 1 per cent phenol in the refrigerator (+10° C.). The results are given in Table IV.

The addition of lecithin enhanced the penetrating power of the disinfectant.

In order to demonstrate the feasibility of the embedding method in testing fungicides the following experiment was performed.

*Experiment 6.*—Cotton strings were infected with *Trichophyton gypseum* according to our formerly described method.<sup>14</sup> One series of these strings is embedded in 1 per cent agar; the covering layer is ca. 2.4 mm. thick. This series



is treated with zephiran, aqueous solution 1:1,000. At the same time another series of the same batch of infected strings is treated directly with zephiran, aqueous solution 1:5,000. Both series are kept at room temperature. The experiment is performed in duplicate. It was necessary to dilute the zephiran for the direct treatment, since preliminary experiments with dilutions 1:1,000 and 1:2,000 respectively had shown that these concentrations killed the fungus within two to five minutes. Table V demonstrates the results.

TABLE IV

	HOURS							DAYS					
	2	5	7	11	24	28	31	2	3	4	5	6	7
Embedded in 3 per cent gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+
Embedded in 3 per cent gelatin containing 0.2 per cent lecithin	+	+	+	+	+	+	+	+	+	+	+	+	+
Direct	+	+	+	+	-	-	-	0	0	0	0	0	0
	DAYS												
	8	9	11	12	13	14	15	16†					
Embedded in 3 per cent gelatin	+	+	+	+	+	+	+	+					
Embedded in 3 per cent gelatin containing 0.2 per cent lecithin	+	+	-	-	-	-	-	-					
Control start +													
Control end. +													
+ = growth of <i>E. coli</i> , identified by subculture													
- = sterile 0 = not done † discontinued after 16 days													

Control start: +

Control end: +

+ = growth of *E. coli*, identified by subculture

- = sterile 0 = not done † discontinued after 16 days

TABLE V

	MINUTES					HOURS			DAYS							
	2	5	10	20	35	1	20	24	2	3	4	5	6	7	8†	
Embedded, zephiran 1:1,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Direct, zephiran 1:5,000	+	+	+	+	-	-	0	0	0	0	0	0	0	0	0	

Control start: + Control end: +

+ = growth - = sterile 0 = not done ‡ discontinued after 8 days

While in the direct experiment the dilution 1:5,000 killed the fungus within 35 minutes, the embedded fungus proved to be viable yet after 8 days though the fivefold concentration of the germicide was applied. The duplicate experiment yielded similar results.

This experiment may have practical applications, as it demonstrates how different it is, whether the fungicide can act directly on the causative agent of athlete's foot or whether it has to penetrate a layer which separates it from the fungus.

It may be mentioned that in experiments with fungi as well as with bacteria some species of microorganisms exhibited peculiarities of growth after having been exposed to the action of a germicide: such as lack of surface growth or delayed surface growth (*Trichophyton gypsum*), formation of a thick pellicle (*Escherichia coli*), delayed pigment formation (*Pseudomonas aeruginosa*). In every instance of such an event the identity of the surviving germs has been verified by subcultures. The causes and conditions of these phenomena are under investigation, and the results will be published in due time.

The described experiments have been chosen to demonstrate the method and its possibilities.

Other experiments have been performed whereby the different factors (kind of germs, medium to be penetrated, etc.) have been varied. Although

we did not try the method on vira, we may assume that the procedure is suitable for testing the penetrating power of viricidal agents. It is furthermore evident that not only liquid germicides but solid (powders) and semisolid (e.g., ointments) disinfectants can be examined as well. Finally it might be mentioned that the method can be adapted for gaseous germicides.

#### SUMMARY

The necessity of testing the penetrating power of germicides has been stressed repeatedly. The existing methods have limited fields of application. A new method is here described. It can be practiced with different kinds of germs (fungi, bacteria, vira) either in pure cultures or in mixtures. The substance to be penetrated can be modified as to material and concentration. Furthermore, the penetrated distance can be appraised, and the temperature can be varied. All kinds of germicides (liquid, solid, gaseous) can be tested. The technique takes into account the retarding effects of deviation and penetration at the same time, simulating hereby natural conditions. As a tentative name, "method of the embedded germ carriers" is suggested.

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## THE USE OF THE ALBINO RAT IN THE PREGNANCY HORMONE TEST\*

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THE original Aschheim-Zondek Test, in which five immature female mice, three to four weeks old, were utilized for the diagnosis of pregnancy was soon replaced by other tests to simplify the procedure. The best-known and most widely used modification at the present time is that of Friedman using rabbits. Most laboratories have become so accustomed to this modification that it is difficult to replace the use of rabbits with other test animals.

The animals which give the most reliable results are the young immature female albino rat or allied strains such as the black-white hooded rat, and the South African frog (*Xenopus laevis*). The method using the frog is rapid, but requires special attention and observation; also this special frog is difficult to obtain at the present time.

Rats have been used for the pregnancy hormone test since 1929, Trivino cited by Eberson and Silverberg.<sup>1</sup> In 1938, Walker and Walker<sup>2</sup> published an article in which they modified the test and reduced the time for observation and diagnosis to thirty hours. They injected 1½ c.c. of urine three times during one day and autopsied the animals about thirty hours after the first injection. Later Frank and Berman,<sup>3</sup> also Kelso,<sup>4</sup> published reports on a twenty-four-hour modification for the diagnosis of pregnancy. Kelso also modified his procedure; this enabled him to read the results eight hours following the injection of 5 c.c. of urine. Salmon and co-workers<sup>5</sup> recently reported a modification reducing the time to six hours. All these authors report between 97 and 100 per cent accurate results in their series of experiments.

It is the purpose of this paper to demonstrate that this test in rats is only reliable if used with caution by experienced personnel and after allowing sufficient time to elapse between the injection and the autopsy.

*Changes Seen in the Rat Ovaries.*—Previous authors give detail description of the appearance of the ovaries following injection of the urine in negative and positive cases. In negative cases the ovaries do not respond to the injected urine and remain small and are cream-colored or pink (Fig. 1A). Colorless follicles may be noted, but these are, according to some authors,<sup>3, 6</sup> of no significance and are considered negative. A positive reaction is characterized by enlargement of the ovaries and marked hyperemia. The ovaries then show

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Since sending in our paper for publication we have been trying out injecting the extract of the gonadotropic hormone in the urine obtained according to the method of Crew. The details of this method are given in an article published by Weisman and co-workers: The Frog Test (*Xenopus laevis*) as a Rapid Diagnostic Test for Pregnancy.

Reference: Weisman, Abner I., Snyder, Arnold F., and Coates, Christopher W.: Am. J. Obst. & Gynec. 43: 135, 1942.

We are not as yet ready to submit our results, but we feel that the use of the concentrated hormone in the urine is of definite advantage.

a deep red color (Fig. 1C). Occasionally hemorrhagic follicles are seen, but it is commonly agreed that this finding is not necessary to determine a positive reaction. Kelso believes that the development of hemorrhagic follicles is only an indication of a stronger reaction. According to Salmon and his co-workers, so-called borderline reactions may occur. Unfortunately this term is not clearly explained by the authors. We assume therefore that they think of a borderline reaction when there is a simple hyperemia of the ovaries, probably without enlargement. It is conceivable that such a reaction may cause confusion and lead to a misinterpretation of the results of the test. We have encountered indefinite reactions in our series of injections (Fig. 1B). This problem will therefore be discussed later in this paper.

#### EXPERIMENTAL STUDIES AND RESULTS

Our experimental study of 100 cases of suspected pregnancy was divided into two groups. Altogether 155 rats were injected. In the first group, two to five animals were given one injection each, with 4 c.c. of urine from the same specimen, and the results were read after two, four, six, eighteen, and twenty-four hours. In the second group the rats were given three injections of 2 c.c. of urine each, according to the method of Walker and Walker using only one animal per test. The results in the rats were observed before control tests using rabbits were performed. The ovaries of the rabbits were then inspected to assure definite orientation as to the reliability of the test in rats. The results are summarized in Chart 1.

*Discussion of Results.* (Chart 1.)—The results in our series reveal several interesting facts. While the inspection of the ovaries six hours after injection of urine gave about 96.5 per cent reliable results, inspection two to four hours after injection gave unreliable readings in a large number of cases. These readings were mostly negative or indefinite reactions showing slight hyperemia but no definite enlargement. Some of the reactions were definitely positive, the ovaries being enlarged and presenting the typical deep red color. When rats injected with the same urine from the positive case were killed after six to eighteen hours, the results were positive in some of those which gave negative results after two to four hours. Some of the rats showing indefinite reactions after two to four hours were found later to be either positive or negative. Only the typical positive reactions found two to four hours after injection could always be confirmed as positive after six hours. All of the injections were confirmed by injecting control rabbits. Cases in which there was disagreement between the results in rabbits and those in rats were analyzed concerning the clinical history.

The second group of rats which received three injections of 2 c.c. of urine each and which were autopsied thirty hours after the first injection showed reliable results in all rats injected. This procedure seems to be the safest for the beginner until he has become familiar with the typical reactions. Of interest are those cases in which there was disagreement between the results in rats and in rabbits. In six cases rats showed positive ovaries while the reactions in the rabbits were negative. Investigation of the clinical history in these cases revealed that we were dealing with cases of early pregnancy. From all six

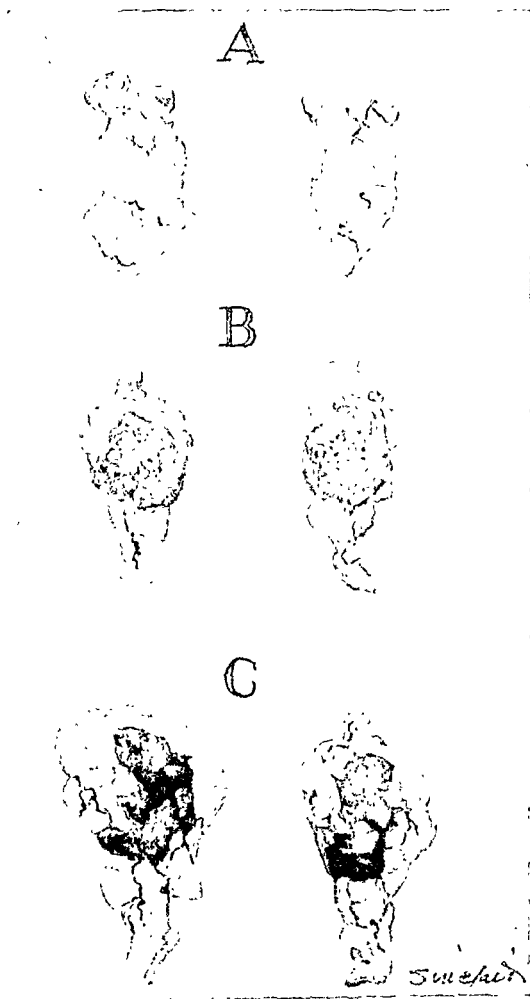


FIG. 1.

cases a second specimen was obtained seven to ten days later. The control rabbits then injected with these urines gave positive reactions.

In seven cases the rats were negative, while the injected rabbits showed positive reactions. Checking these cases revealed that none of the women were pregnant. We were dealing therefore with false positive reactions in rabbits. It is generally believed that these false positive results in rabbits are due to improper segregation and overage of the animals; however, experience has shown that even with preliminary operative inspection of the ovaries, occasionally false reactions are observed. Especially interesting in this respect is the report of Davy and Sevringhaus,<sup>7</sup> who encountered a relatively large margin of error when the mature female rabbit test was used. These authors point out all the conditions which may lead to false negative and false positive results and come to the conclusion that false positive results are rather rare when the immature female rat or rabbit is used and that false negative results are less frequently

## RESULTS OBTAINED

*AT ST. VINCENT'S HOSPITAL. TOLEDO, OHIO.*

*URINE SPECIMENS EXAMINED FOR SUSPECTED PREGNANCY  
USING RATS AND CHECKED BY RABBIT INJECTION.....100*

*URINE POSITIVE IN RATS AND RABBITS.....56*

*URINE NEGATIVE IN RATS AND RABBITS.....31*

*DISAGREEMENT IN RESULTS IN RATS AND RABBITS.....13*

*EXPLANATION OF DISAGREEMENT IN RESULTS*

*EARLY PREGNANCY SIX CASES. RATS (+), RABBITS (-).*

*THIS WAS CHECKED BY INJECTION OF RABBITS TWO WEEKS  
AFTER FIRST TEST. RABBIT THEN POSITIVE.*

*FALSE POSITIVE RABBITS SEVEN CASES. RATS (-), RABBITS (+).*

*THESE FALSE POSITIVE FINDINGS IN RABBITS DUE TO  
IMPROPER SEGREGATION OR OVER AGE OF RABBITS  
PURCHASED FROM UNRELIABLE DEALERS.*

*COULD BE AVOIDED BY PRELIMINARY OPERATIVE  
INSPECTION OF RABBITS.*

*RESULTS OBTAINED AS OBSERVED IN DIFFERENT TIME  
INTERVALS.*

*RATS INJECTED.....155*

*14 AFTER 2 HOURS NO DIAGNOSIS POSSIBLE.....8*

*22 AFTER 4 HOURS NO DIAGNOSIS POSSIBLE.....4*

*28 AFTER 6 HOURS NO DIAGNOSIS POSSIBLE.....1*

*46 AFTER 18 to 24 HOURS DIAGNOSIS POSSIBLE.....46*

*45 AFTER 36 HOURS DIAGNOSIS POSSIBLE.....45*

Chart 1.

seen in rats in cases of early pregnancy. Our results confirm the findings of these authors. We cannot emphasize strongly enough, however, that considerable experience is necessary in reading the results of the tests in rats. An extremely safe technique must be applied, and in all doubtful cases the history should be analyzed and the test repeated.

Occasionally indefinite reactions were encountered in the form of slight hyperemia, but by keeping to the rule that positive reactions show a marked

hyperemia and enlargement and that the hyperemia is of a deep red color and that negative reactions do not show the marked hyperemia or enlargement, also by injecting another test animal whenever in doubt, seldom a diagnosis was made contrary to a control test in rabbits. We have kept to this routine in all additional cases which we have observed subsequent to this primary study. In the later cases rabbits were not available for checking all our results, but we have encountered only an occasional difficulty. Checking the history in these few cases giving indefinite reactions, we obtain the impression that uncertain reactions may occur when fetal or maternal pathology is present. These instances are the exception rather than the rule, and in general the results obtained are satisfactory. We repeat the test whenever we are in doubt or whenever the slightest suggestion is made by the clinician that there might be some discrepancy between our findings and the clinical diagnosis. Injection of another animal after a few days as a rule gives definite orientation whether pregnancy exists or not.

#### ADVANTAGES AND DISADVANTAGES

The results just described and analyzed confirm the opinion that the rat is a suitable animal to replace the rabbit and that errors in diagnosis are few. In addition, the test consumes less time (six to thirty hours). The cost of the animal is much less. The rat can tolerate relatively more urine, and detoxification is not as essential. From our 155 rats only one animal ( $\frac{1}{2}$  per cent) died following injection, while from 100 rabbits three animals (3 per cent) died. The greatest disadvantage is based on the fact that one must become familiar with the typical negative and the typical positive reaction. Caution must be taken that a slight hyperemia is not interpreted as a positive reaction. The age of the rats is limited to from twenty-two to forty days. Proper selection of the female animal for the test is necessary. Careful observation of the animals during the time of mating and breeding is essential, and correct records must be kept, when a rat colony is started. (Rat Record Sheet, Chart 2.)

#### RECOMMENDATIONS FOR THE USE OF RATS IN THE PREGNANCY HORMONE TEST

Realizing that it is extremely difficult to replace a well-established method with another less known procedure, but considering the scarcity of rabbits in many areas of the country, we could not consider this paper to be complete without giving some suggestions concerning the technique, possible errors, and the source of the animals.

1. *Technique (Fig. 2).*—The principal steps of the technique are shown in Figs. 2 and 3. These need no further explanations. Before injecting the animal, however, one should make certain that a female animal is injected. Fig. 3 shows the differentiation between the male and female animal.

In regard to the injection of the urine, we usually give one injection of 4 c.c. and perform the autopsy at any time between six and eighteen hours, following the injection. In some cases we use a slight modification of Walker and Walker's method using two injections of 2 c.c. of urine on one day and another injection of 2 c.c. the following morning. The animal is then autopsied six hours after the last injection. This procedure, however, requires more time, and if one is desirous of an early diagnosis, the one injection method using  $\frac{1}{2}$  c.c. is satisfactory.

As a rule we use one animal for each test. When uncertain reactions occur, the test is repeated. In any case we consider one injection of 2 c.c. of urine not sufficient to be reliable.

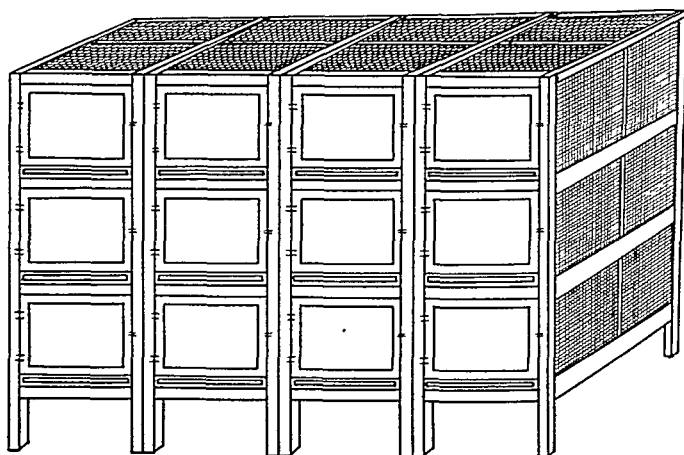
2. *Sources of Errors.*—Unreliable results are mostly due to negligence in performing the test and to improper reading of the results in the ovaries. These mistakes can be prevented. False negative or positive reactions due to pathologic conditions in the pregnant or nonpregnant women cannot be prevented, but an analysis of the clinical histories in these cases somewhat eliminates a wrong conclusion. In order to eliminate preventable errors as much as possible, the sources of these errors are discussed here briefly:

### RAT CAGE - RECORD SHEET

ST. VINCENT'S HOSPITAL TOLEDO 7, OHIO

#### SMALL RAT COLONY

CHART 2



REMARKS:

DIETARY REQUIREMENTS:

Chart 2.

A. *The Urine.*—The specimen should not be obtained too early after the menstruation period failed to appear. The specific gravity should be above 1010. The reaction should be neutral or slightly acid; if alkaline, it should be acidified with a few drops of a 50 per cent acetic acid solution. The specimen should be clear and free of albumen. Specimens with heavy bacterial contamination or foul odor should be rejected. One should insist on obtaining



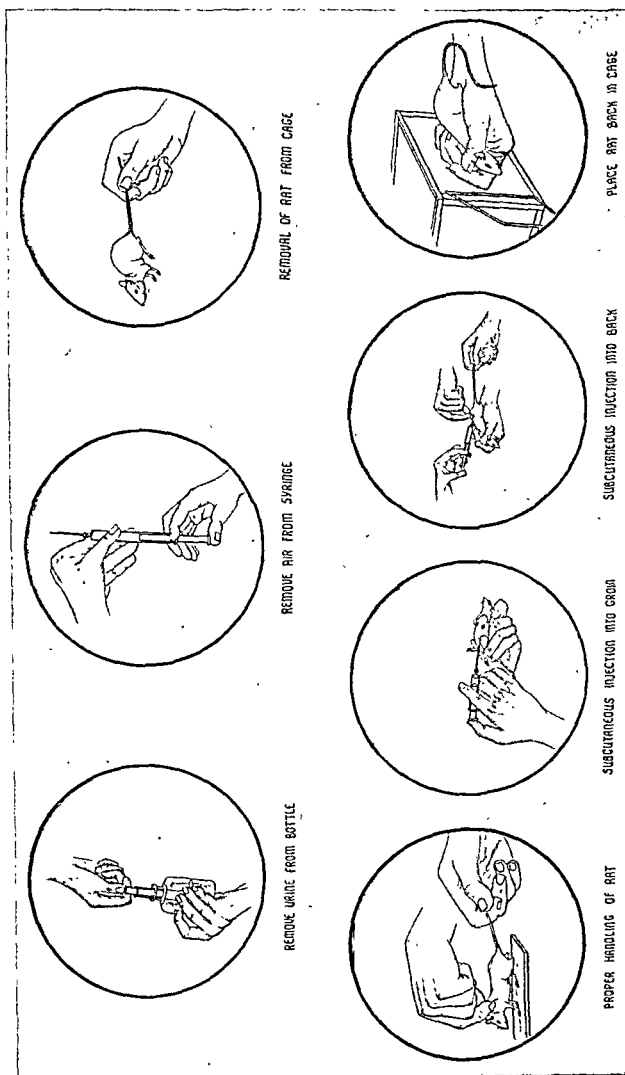
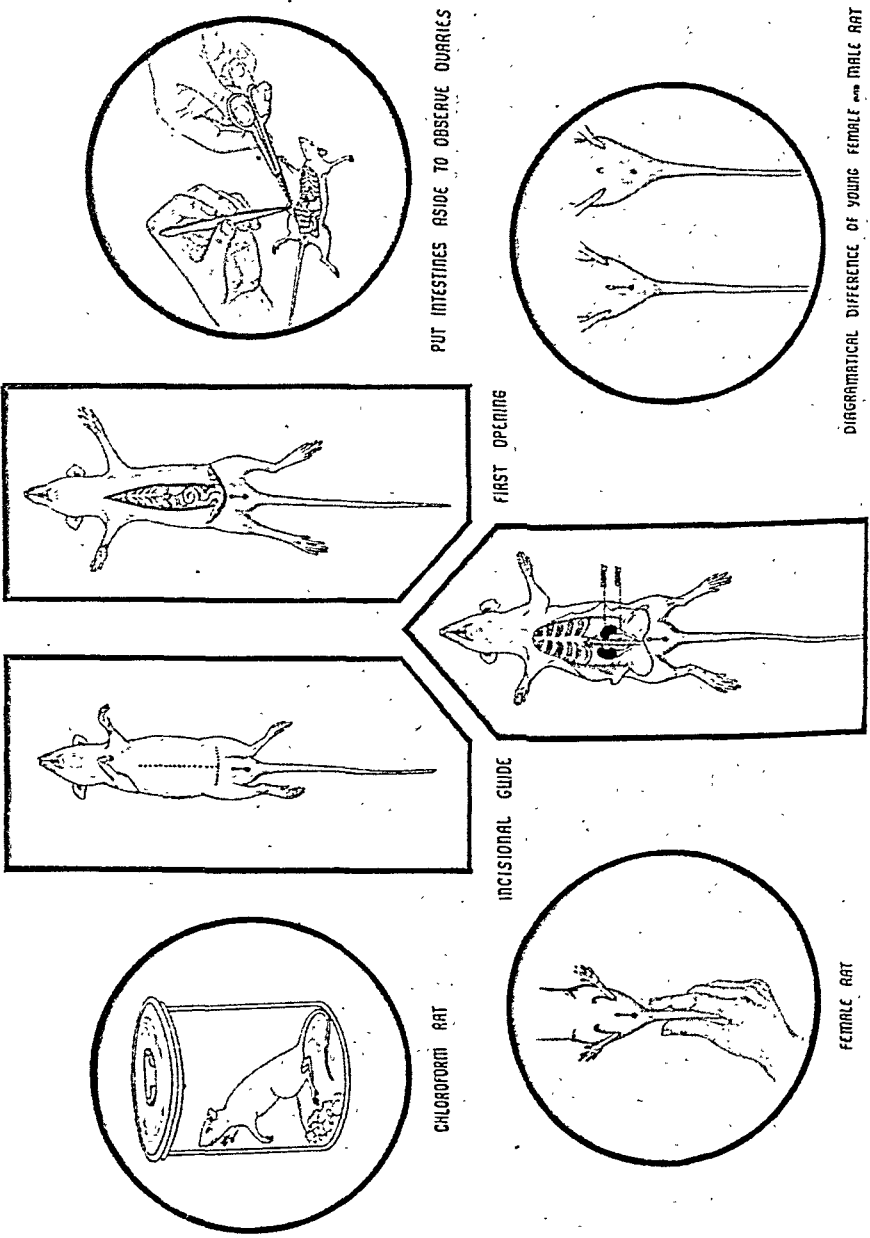


FIG. 2.



ANATOMY OF RAT, POSITION OF OVARIES  
FIG. 3.

a fresh morning urine. Fluids should be restricted after 8 P.M. the night before the urine is obtained. According to Davy and Nason,<sup>8</sup> boric acid is the best preservative if the specimen cannot be kept in the icebox until used.

*B. Points Concerning the Use of the Animals.*—The rats used for the test must be properly selected. The weight of the animal should be between 35 and 40 grams, the age between 22 and 40 days. We have used rats up to 45 days of age with reliable results. Rats older than 45 days and younger than 22 days should not be used. Animals derived from a large litter are usually underdeveloped. Such animals should not be used at the age of 22 days, but can be used if about 30 days old, provided that their nutritional status then is good. If underdeveloped animals are used, their ovaries might be refractory for a satisfactory response. The question of proper nutrition is of great importance for raising of a good litter. The test animals must be kept away from the male animals. Noise and excitement should be avoided as well as misplacing the animals.

*C. The Autopsy (Fig. 3).*—The autopsy should not be performed too early after injection for the reasons outlined previously. The ovaries must be inspected immediately after the rat is killed. Chloroforming of the animal and inspection of the ovaries should be a coherent step in the procedure, not taking longer than four to five minutes. If this is not done, the most important sign of a positive reaction, hyperemia, may disappear quickly, and a false negative interpretation may be made.

*D. Reading of the Ovaries.*—The appearance of the ovaries in negative, positive, or indefinite reactions has been discussed previously. It is absolutely necessary to become familiar with these reactions. Typical negative and typical positive reactions are so distinct there should be no difficulty in diagnosis. Indefinite reactions, however, are difficult to interpret and may be responsible for a false diagnosis. In such cases it is best to inject another animal. The appearance of colorless follicles without hyperemia or enlargement should be considered a negative reaction.

*3. Source of Rats for the Test.*—The animals can be obtained from commercial dealers or can be raised in a rat colony. A very useful book on this subject is that by Main.<sup>9</sup> Sufficient space, clean quarters and cages, and a vitamin rich diet are necessary. Interbreeding of new strains of rats should take place at certain intervals. The greatest difficulty in keeping up the rat colony is the proper timing of matings, because of the difference among the animals in their physiology of pregnancy. Another difficulty is the instability of the number of males and females in one litter. We, therefore, buy some rats from a reliable dealer whenever it is necessary.

#### SUMMARY

The advantages and disadvantages of the pregnancy hormone test in rats are discussed, and it is pointed out that many technical errors may be encountered. Further studies are needed to make this test 100 per cent reliable. Concentration of the urine specimens may be an aid in the solution of some of our difficulties; however, we feel that this test can be used at the present time and particularly so because of the difficulty in obtaining rabbits. With the precautions applied as described, we firmly believe that the test using rats

is not less reliable than when performed using rabbits. Our study shows errors may be encountered, but they may also be encountered when rabbits are used. We feel that the enthusiasm of previous authors in regard to the substitution of rats for rabbits in this test is somewhat justified.

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# CHEMICAL

## A MICROMETHOD FOR THE DETERMINATION OF ETHYL ALCOHOL IN BLOOD\*

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**B**ECAUSE of the need for a rapid and yet accurate method of determining ethyl alcohol in blood taken from hospital patients for diagnostic purposes and from the deceased for medicolegal evidence, a micromethod with which two determinations may be run simultaneously has been devised and experimentally found to be highly satisfactory. Its merit lies largely in the fact that the method is valid over approximately twice the range of blood alcohol concentrations as is determinable by previous methods. The necessity of the extension of this range becomes apparent from our experience that in both living and dead individuals with a clear acute alcoholic history blood alcohol concentrations have attained 0.7 per cent. All the previous methods cited below fail to exhibit evidence that their blood alcohol concentrations are valid above 0.4 per cent for a given blood analysis sample.

In addition, the method described in this paper uses solutions which are stable indefinitely, requires but a few drops of blood (0.1 c.c.), and involves a simplified method of obtaining the final per cent of alcohol. However, no claim is made for the specificity of the method, inasmuch as the analysis of blood taken from alcoholic individuals given paraldehyde as a sedative proved to be absurdly high. Usually this difficulty is removed by the withdrawal of the blood sample prior to medication.

Widmark<sup>1</sup> in 1922 appears to have been the first to reduce the determination of ethyl alcohol in blood to micro dimensions. Many papers modifying the Widmark method followed, among the more important of which were the works of Aoki,<sup>2</sup> Galamini and Bracaloni,<sup>3</sup> Koller,<sup>4</sup> Kaiser and Wetzel,<sup>5</sup> Heiduschka and Flotow,<sup>6</sup> Holzer,<sup>7</sup> Kanitz,<sup>8</sup> Soltan,<sup>9</sup> Mayer,<sup>10</sup> Cavett,<sup>11</sup> Stempel,<sup>12</sup> du Pan,<sup>13</sup> and Winnick.<sup>14</sup>

Other methods were developed, some entirely new in principle, others, micromodifications of former macromethods. These were investigations by Hiramatsu,<sup>15</sup> Abels,<sup>16</sup> Levine and Bodansky,<sup>17</sup> Shapiro,<sup>18</sup> Heiduschka and Steulmann,<sup>19</sup> Liebesny,<sup>20</sup> Anderson,<sup>21</sup> Decker,<sup>22</sup> Nieloux,<sup>23</sup> Johnston and Gibson,<sup>24</sup> Fish and Nelson,<sup>25</sup> Newman and Abramson,<sup>26</sup> Kozelka and Hine,<sup>27</sup> Goubau,<sup>28</sup> and Thivolle and Sonntag.<sup>29</sup>

### REAGENTS

1. Sodium tungstate ( $\text{Na}_2\text{WO}_4$ ) solution  
(10 per cent aqueous solution)

\*From the Cook County Coroner's Chemical Laboratory.  
Received for publication, Oct. 30, 1943.

## 2. Mercuric sulfate solution

50 Gm. of mercuric sulfate ( $\text{HgSO}_4$ ) and 28 c.c. of concentrated sulfuric acid are diluted to 500 c.c.

## 3. Potassium iodide solution

40 Gm. of potassium iodide (KI) per liter.

## 4. Potassium dichromate solution

8.3903 Gm. of potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ , Mallinckrodt, Analytical Reagent Grade)\* is diluted to 2000 c.c. of solution (0.08555 N. as oxidizing agent with respect to ethyl alcohol.)

## 5. Sodium thiosulfate solution.

Approximately 4.7 Gm. of sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ), 1.0 Gm. sodium hydroxide, and 0.5 Gm. benzoic acid are diluted to 1 liter of solution. This solution is filtered prior to use.

## 6. Starch solution

2 Gm. soluble starch per liter of water.

This solution is preserved indefinitely if kept in refrigerator when not in use.

## PROCEDURE

A 0.1 c.c. Normax pipette (to contain 0.1 c.c.), after having been cleansed with dichromic-sulfuric acid solution and washed several times with distilled water, is rinsed twice with the homogenous blood to be analyzed. The blood is then drawn up about 5 mm. beyond the mark, and then cautiously lowered to the mark by the application of a piece of cloth to the end of the pipette which removes the blood by blotting action.

The pipette containing the blood is then transferred to the distillation flask, care being exercised to prevent any blood from touching the sides of the flask. The pipette is then washed three times with distilled water (both inside and out). One c.c. each of the sodium tungstate and mercuric sulfate-sulfuric acid solutions are added and the flask sides then rinsed with distilled water.

The distilling heads connecting the distilling flasks and the condensers are then put into place. (See Fig. 1.)

The distillate receiving flasks are 50 c.c. Erlenmeyers to which had been added 1.000 c.c. of standard dichromate solution from a 1 c.c. microburette and 4 c.c. of chemically pure grade concentrated sulfuric acid (Mallinckrodt) delivered from a 5 c.c. microburette.† These, after being cooled, are placed in such position that the condenser tip is approximately 2 cm. above the dichromate solution. These mixtures, if kept in the sunlight, must be used within a few hours, inasmuch as the sulfuric acid contains an impurity, perhaps dissolved sulfur dioxide, which reduces the dichromic acid in the acid mixtures at room temperature to the extent corresponding to approximately 0.003 per cent ethyl alcohol per hour. Further experiments showed that the rate of reduction of the dichromic acid in complete darkness was found to correspond to 0.0005 per cent alcohol per hour. This phenomenon is not new, having been observed

\*Potassium dichromate crystals were pulverized, oven-heated at  $110^\circ \text{C}$ . for several hours, and then vacuum-dried over phosphorus pentoxide prior to use.

†Both the 1 c.c. burette (for dichromate) and the 5 c.c. burette (for the sodium thiosulfate) were Koch-type microburettes obtainable from the Scientific Glass Company, Bloomfield, N. J.

previously.<sup>30</sup> If not used immediately, these solutions should be placed in the dark where the dichromate reduction is negligible over a 24-hour period.

The steam flask, not yet connected to the distillation flasks beyond the T tubes, is then heated, and the developed steam is permitted to wash out the air in the entire steam system, the steam of course, escaping beyond the T tubes into the air. The steam-generating system is then connected with the distilling flasks (if one blood sample is analysed, one of the screw clamps is adjusted to close the lumen of the rubber tubing), and after steam is permitted to flow out of the vertical portion of the T tubing for several seconds, this flow is stopped by closure of the pinch clamps.

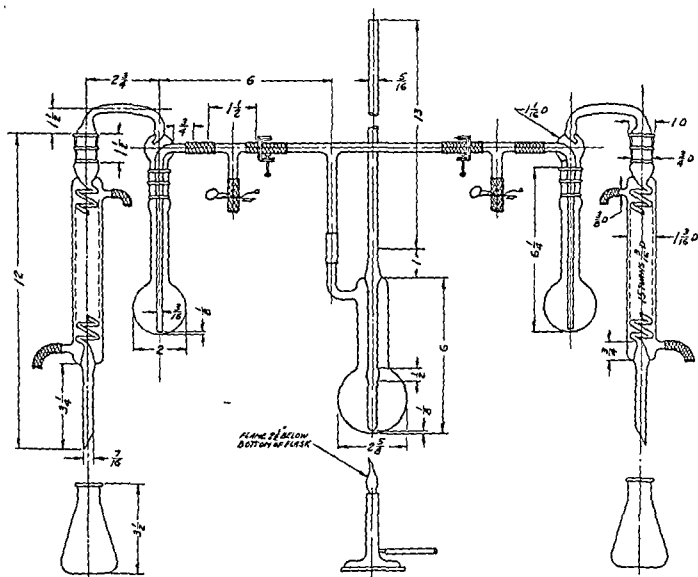


Fig. 1.

The flame is adjusted to permit the rate of distillation to be such that about 70 drops of distillate are formed, or the 8.5 c.c. mark is reached in approximately five minutes after which the pinch clamps on the T rubber tubing are opened and the flame removed. This volume should not be exceeded as all the alcohol will have been distilled over into the dichromic acid mixture when this volume will have been reached, and any further distillation unnecessarily dilutes the alcohol-dichromic acid mixture leading to incomplete reaction with a resulting low alcohol value.

The flasks are covered with inverted 30 c.c. Erlenmeyers and the contents mixed well by a rotary movement. Each, after the inverted flask is removed, is

then placed in the steam bath\* for twenty minutes, after which time they are withdrawn and cooled under the water tap. One c.c. of the potassium iodide solution is pipetted into the contents of the flask which is then mixed well by vigorous rotation of the flask. About 2 c.c. of distilled water are then added, and the contents are titrated with standard sodium thiosulfate solution, the flask being rotated vigorously during the titration. When the solution becomes a faint yellow in color, 4 drops of starch solution are added, and, after the contents are mixed, the titration is completed to a green-tinted almost colorless end point, the final drops being fractionated on the tip of the burette.

Blank mixtures containing 1 c.c. of the standard dichromate and 4 c.c. of the concentrated sulfuric acid are occasionally subjected to the procedure (heated on steam bath, etc.) in order to check both the reagents and the blank titer, the latter being necessary for the construction of the graph from which the per cent alcohol is read.

#### CALCULATION

If:

B = Titer of blank in c.c. of sodium thiosulfate

T = Titer of reduced acid dichromate mixture (by blood distillate) in c.c. of sodium thiosulfate

$N_k$  = Normality of potassium dichromate solution

$N_s$  = Normality of sodium thiosulfate solution =  $\frac{N_k}{B}$

P = Per cent ethyl alcohol in blood sample by weight

And if:

1.050 Gm. per c.c. is assumed to be the average density of blood, and ethyl alcohol is oxidized under the procedure conditions to acetic acid (i.e., 4 electrons are lost per molecule of ethyl alcohol),

Then:

$$P = \frac{46}{4} \frac{(B - T) (N_s) (100)}{(1000) (0.105)}$$

This reduces to:

$$P = \frac{46}{4.2} (B - T) (N_s)$$

Substituting:

$$N_s = \frac{N_k}{B}$$

And letting:

$$\left( \frac{46}{4.2} \right) (N_k) = C$$

Then:

$$P = C \left( \frac{B - T}{B} \right) = C \left( 1 - \frac{T}{B} \right)$$

\*The steam bath consisted of an iron dish in which several 250 c.c. beakers were placed. Both the dish and the beakers were half filled with distilled water, and the whole dish was heated with a flame until the boiling point of water was reached. Each beaker conveniently accommodated one Erlenmeyer flask with no danger of tipping, and thus several flasks could be heated on the steam bath simultaneously.



$$P = -\frac{C}{B}(T) + C$$

This is an equation of a straight line with slope  $= -\left(\frac{B}{C}\right)$  and with a

T - axis intercept of B and with a

P - axis intercept of C

Hence, if, on a large graph paper, the two axis intercepts are plotted as in Fig. 2, and a straight line drawn through them as indicated, then the per cent blood alcohol by weight can be obtained quickly for any sodium thiosulfate titer by the use of a celluloid right triangle as diagrammed. The accuracy of the method for varying amounts of ethyl alcohol is shown in Table I.

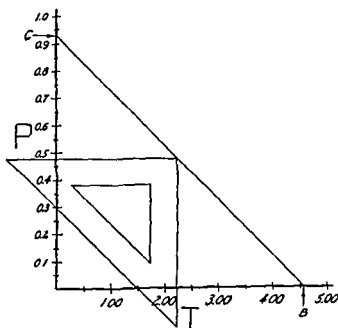


Fig. 2.

TABLE I

COMPARISON OF EXPERIMENTALLY DETERMINED AND TRUE PER CENT ALCOHOL BY WEIGHT IN HUMAN BLOOD AS OBTAINED BY MICROMETHOD

TRUE VALUE*	FOUND	FOUND	FOUND
Bank Blood	0.002	0.002	0.005
0.12	0.13	0.14	0.13
0.23	0.23	0.25	0.25
0.35	0.37	0.36	0.36
0.46	0.47	0.45	0.45
0.59	0.61	0.60	0.60
0.74	0.72	0.72	0.73
0.88	0.86	0.88	0.86

\*Absolute alcohol (prepared by the distillation of commercial absolute alcohol after it had been treated with sodium amalgam) of appropriate order of weight was added to approximately 10 c.c. of blood in glass-stoppered weighing bottles. Weighing by difference led to the known weights of alcohol and blood from which the per cent alcohol by weight was calculated. These mixtures were well shaken before their analysis.

#### SUMMARY

1. A micromethod is described in which the alcohol concentration in bloods containing up to 0.88 per cent alcohol can be determined within an average of 5 per cent error. This range is approximately twice that determinable by any previous micromethod from a given blood sample.

2. The method requires only a few drops of blood (0.1 c.c.) which a finger or ear prick could provide, venous puncture being unnecessary.
3. The method involves the use of reagents which are stable indefinitely.
4. A simplified calculation is derived and described, minimizing the possibility of arithmetical error and permitting the final per cent alcohol to be obtained quickly.
5. The method is not time-consuming, permitting the analysis of two blood samples simultaneously in about thirty-five minutes.

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# THE ADAPTATION OF THE DIRECT BIURET METHOD FOR THE DETERMINATION OF SERUM PROTEINS TO BOX COMPARATOR COLORIMETRY\*

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A S photoelectric colorimeters are nonstandard army hospital field equipment, and a Duboseq type colorimeter is impracticable for portable field kits, the direct biuret method<sup>1</sup> for the determination of serum proteins was adapted to the box comparator type of colorimetry. The box comparator was made from a 2 by 4 inch length of soft pine wood. Holes were bored for seven pairs of  $16\frac{1}{2} \times 150$  mm. matched Pyrex tubes for racking, and for matching three single holes with  $14 \times 35$  mm. centered upright intersecting apertures cut through the sides of the block. A thin glass green light filter with maximum transmission at  $540 \mu$  should be used if available. A thin frosted piece of glass or paraffin oiled sheet of thin copy paper may be attached over the apertures to the side of the box. Fairly good matching of the color tubes was obtained in a good north light without light filters.

## REAGENTS

*Biuret Reagent (T.P.) for Total Protein.*—To 500 c.c. of 14 per cent sodium hydroxide ("carbonate free") in a Pyrex bottle or flask, add 100 c.c. of 1 per cent copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) solution and mix. Protect from dust and other contaminants. A rubber stopper may be used.

*Biuret Reagent (ALB) for Albumin.*—To 300 c.c. of 23 per cent sodium hydroxide ("carbonate free") in a Pyrex bottle or flask, add 100 c.c. of 1 per cent copper sulfate solution and mix.

*Ether.*—U.S.P. grade is satisfactory.

*Sodium Chloride.*—0.9 per cent.

*Sodium Sulfate.*—23 per cent.

*Standard Serum (for Calibration).*—Obtain 5 to 10 c.c. of a single or mixed specimen of normal serum free from cells and hemolysis. Determine the protein concentration in duplicate samples by the macro- or micro-Kjeldahl method.

## PREPARATION OF STANDARD BIURET TUBES FOR MATCHING IN BOX COMPARATOR

*Preparation of Biuret Stock Standard.*—Prepare an 8.4 per cent stock standard by adding 5 c.c. of the standardized serum to X

$$\left( \frac{\text{per cent T.P. of serum} \times 300}{8.4} \right)$$

c.c. of biuret reagent (T.P.).

Shake vigorously the stock standard with 50 c.c. of ether for five minutes and let stand forty-eight hours in a glass-stoppered cylinder. Carefully pipette off the solution to be used for standard from beneath the ether layer and avoid mixing the small amount of lipoidal precipitate which forms between the water and ether layers.

\*From the Laboratories, McCloskey General Hospital, Temple, Tex.  
Received for publication, Jan. 3, 1944.

*Preparation of Standard Color Comparison Tubes From Stock Standard.*—Prepare standard tubes from 8.4 to 3.2 per cent at intervals of 0.4 per cent (Table I).

For Example: to prepare an 8.0 per cent standard:

$\frac{8.4}{8.0} \times 6.0 = 6.3$ .  $6.3 - 6.0 = 0.3$  c.c. of biuret reagent (T.P.) to be added to 6 c.c. of stock standard to prepare an 8.0 per cent standard tube. Uniform matched Pyrex test tubes should be used for protein determinations and standard tubes. Tubes should be stoppered tightly with paraffin-coated corks or rubber stoppers.

TABLE I  
VOLUME OF REAGENTS FOR PREPARATION OF STANDARD TUBES

PROTEIN STANDARD PER CENT	C.C. OF 8.4 PER CENT OF STOCK STANDARD	C.C. OF BIURET REAGENT (T.P.)
8.4	10	0.0
8.0	9	0.45
7.6	9	0.96
7.2	9	1.50
6.8	9	2.11
6.4	9	2.81
6.0	9	3.60
5.6	6	3.00
5.2	6	3.70
4.8	6	4.50
4.4	6	5.45
4.0	6	6.60
3.6	4	5.33
3.2	4	6.50

#### METHOD

*Total Protein.*—To exactly 6 c.c. of biuret reagent (T.P.) in a standard colorimeter tube, add 0.1 c.c. of fresh serum (free from cells and hemolysis) with a micropipette, after wiping the outside of the pipette. Rinse the pipette three or more times with the biuret reagent. Add 2 c.c. of ether, stopper, shake vigorously for approximately ten seconds, remove the stopper, and immediately centrifuge at about 2,500 revolutions per minute for five minutes. Read against standard tubes in the box comparator within five minutes after centrifuging.

If the serum is clear and contains only traces of hemoglobin and no more than normal concentrations of other pigments, fairly accurate results are obtained without ether extraction and centrifugation if readings are made five minutes after the development of the biuret color. When ether is not used, carefully mix the solutions by inverting; avoid shaking.

*Albumin.*<sup>2</sup>—Add 0.5 c.c. of serum to 7.5 c.c. of 23 per cent sodium sulfate in a test tube (18 by 120 mm.) and mix thoroughly by twirling. Add about 3 c.c. of ether, rubber stopper, and shake vigorously for twenty to thirty seconds. Unstopper the tube and centrifuge five to ten minutes at about 2,500 r.p.m., or until globulin precipitate separates if hand centrifuge is used. After centrifugation, slant the tube so that the tightly packed globulin precipitate separates from the walls of the tube. Insert a pipette through the ether layer, preferably along the lower wall, withdraw 2 c.c. of the albumin solution, add to 4 c.c. of the biuret reagent (ALB) in a standard colorimeter tube, and mix immediately. Then add 2 c.c. of ether and proceed as in the

biuret method for total protein. Under certain conditions the use of ether may be omitted as stated above. To calculate the amount of albumin present, determine the protein equivalent of the standard tube to which it is matched and multiply by 0.784.

*Globulin*.—Total protein minus albumin equals globulin.

#### DISCUSSION

Different observers were able to obtain readings which checked within a range of 0.0 to 0.3 per cent protein. However, individuals who may be partially color-blind to pink and purple colors should not use this method. The method has been used satisfactorily for the past ten months. The standards were stable for about six months if kept in the dark at room temperature. However, it is believed that refrigerated standards may keep even longer.

#### SUMMARY

An adaptation of the direct biuret method for the determination of serum proteins to box comparator colorimetry has been described. The method has been satisfactory as a substitute for more elaborate equipment.

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## ON THE OCCURRENCE OF ARSENIC IN HUMAN HAIR AND ITS MEDICOLEGAL SIGNIFICANCE\*

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THE keratin tissues in chronic arsenical poisoning are of special importance from a medicolegal point of view, because they appear to fix and concentrate arsenic from the systemic circulation.<sup>1</sup> Thus hair and nails have been frequently analysed posthumously as a means of detecting arsenic administered with homicidal intent.<sup>2</sup> It is therefore of great importance to establish the concentration of arsenic present in normal human hair. This has been done by several investigators using various techniques with very different results as reviewed by Wührer.<sup>3</sup> One to 3 mg. per 100 Gm. of hair is the usual concentration of arsenic in acute and chronic poisoning, but it may be as low as 0.1 mg. Althausen and Gunther<sup>4</sup> record two analyses: 0.08 and 0.16 mg. for normal hair of known history. Wührer<sup>3</sup> carried out thirty analyses on hair from barber shops in Berlin, the great majority of which were between 0.015 and 0.38 mg.

Several other investigators have published figures of this order of magnitude. The results of Myers and Cornwall<sup>5</sup> are much more variable, ranging from 0.00 to 10.6 mg. It therefore appeared to us as a question for further investigation, with the primary object of devising a standard procedure for treating the hair prior to analysis. Our initial conception was that of arsenic from the blood being deposited as a keratin complex in the hair follicle and extending along the shaft with growth. In addition, we pictured the contamination of the hair with arsenical compounds from external sources such as dust or pomades. Our experiments have been designed to remove "external" arsenic by suitable solvents from the "internal" arsenic-keratin complex and thus to arrive at a true normal figure for hair representing the ingestion of arsenic in food or its inhalation.

### EXPERIMENTAL

*Methods.*—Arsenic was determined by the micro-Gutzeit apparatus<sup>6</sup> using Hanford-Pratt strips impregnated with 2 per cent alcoholic mercuric bromide for amounts below 0.02 mg.  $As_2O_3$  and  $\frac{1}{2}$  per cent for higher values. The zinc (granular, 20 to 30 mesh) and hydrochloric acid were the special grades for this purpose. The determinations were carried out in a water bath at  $30^\circ \pm 1^\circ$  C. Washed sand was used instead of the usual roll of absorbent cotton following the directions of Jacobs.<sup>7</sup> Twenty per cent lead acetate was added, and compressed air was passed through the tube for about thirty seconds. Before each determination, the tube to contain the indicator strip was washed with concentrated  $HNO_3$ , rinsed with water, and dried. A standard series of stains from 1 to 20  $\mu g.$  in strips of 1  $\mu g.$  was prepared, using a solution of resublimed

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As<sub>2</sub>O<sub>3</sub> (Merck reagent). The standard at 0.5  $\mu$ g. gave a very slight stain. Blank controls were negative compared with the weakest standard. For values above 20  $\mu$ g., an interval of 0.2  $\mu$ g. was used for the standards. The length of stain on both sides of the paper was measured, averaged, and compared with a curve plotted from the standards.

We have also used successfully the modification of the Gutzeit method with No. 24 cabled cotton thread instead of paper strips for the lowest arsenic values.<sup>8</sup>

Organic matter was removed, either by "dry" ashing with magnesium nitrate according to the Strzyzowski procedure<sup>9</sup> or "wet" ashing with concentrated nitric and sulfuric acids and 30 per cent hydrogen peroxide.<sup>10</sup> Most of the determinations were done by the former method. In this, 5 c.c. of saturated Mg(NO<sub>3</sub>)<sub>2</sub> were used to about 4 Gm. of hair, in a silica crucible, adding enough distilled water to ensure complete wetting. After complete ignition, enough water was added to make a thick paste and then concentrated HCl to about pH 6 by alk-acid paper. Three c.c. of concentrated HCl were then added, and the solution was washed into the apparatus with 25 c.c. of water.

In "wet" ashing, 15 c.c. each of concentrated HNO<sub>3</sub> and H<sub>2</sub>SO<sub>4</sub> were added to 4 Gm. of hair in a 250 c.c. Kjeldahl flask and heated to boiling. Concentrated HNO<sub>3</sub> was added dropwise from a funnel until the solution was nearly colorless, requiring about 50 c.c. in one-half to one hour. After evaporation to about 5 c.c., 3 c.c. of 30 per cent H<sub>2</sub>O<sub>2</sub> were added, and evaporation continued to about 3 c.c. The solution was then made up in a small graduate cylinder to 4 c.c. with concentrated H<sub>2</sub>SO<sub>4</sub>, and the contents of both flask and graduate were washed into the Gutzeit apparatus with 25 c.c. of water. This method gave results that were 5 to 10 per cent higher than the Strzyzowski procedure.

*Extraction With Solvents.*—Several large specimens of human female hair of known history were used in the following experiments: One specimen was extracted with distilled water for forty-one hours, and 3 Gm. samples were taken at intervals for analysis. Forty-seven per cent of the original arsenic present was removed in twelve hours, and it was not increased by more prolonged extraction. Further decreases in the original concentration in the hair were effected with 0.1 per cent NaOH, 0.2 per cent Na<sub>2</sub>CO<sub>3</sub>, and 0.1 per cent HCl for twelve hours each at 20° C. The results on two specimens of hair are shown in Table I.

A decrease comparable to that obtained with NaOH was brought about by extraction of the hair with ethanol or diethyl ether for eight hours. Combining the two procedures gave a further decrease, as shown by Sample 3 in Table I. By way of increasing the speed of extraction, this was carried out under reflux for two hours at 100° and compared with the effect of sixteen hours at 20°. The whole procedure as shown in Table II consisted of initial extraction with alcohol in a Soxhlet apparatus for eight hours, followed by treatment of this material as separate samples with HCl or NaOH or both at 20° and at 100° as indicated. Longer extraction than two hours with HCl and NaOH at 100° did not appreciably decrease the concentration of arsenic. During this period, however, there was an appreciable reduction in free acid and base, 0.1 per cent NaOH becoming 0.04 per cent and 0.1 per cent HCl becoming 0.06 per cent in two hours; this was due to combination with the protein of the hair.



It would thus seem that about 80 to 90 per cent of the arsenic normally present in hair can be removed without destroying the structure of the hair. The remaining 10 to 20 per cent appears to be fixed in such a manner as to suggest possible internal deposition corresponding to the conception of "normal" arsenic. Using the above method of hot extraction with ethanol, NaOH and HCl, determinations have been carried out on several samples of hair of known history carefully collected. The untreated specimens varied from 0.09 to 0.48 mg. per 100 Gm. dry hair, and the treated from 0.02 to 0.05 mg. as shown in Table III. The latter values agree well with those recorded in the literature for the most part. They tend to support our conception of "internal" and "external" arsenic in hair.

TABLE I  
EFFECTS OF AQUEOUS SOLVENTS

TREATMENT	SAMPLE 1		SAMPLE 2		SAMPLE 3	
	As <sub>2</sub> O <sub>3</sub> mg.	DECREASE %	As <sub>2</sub> O <sub>3</sub> mg.	DECREASE %	As <sub>2</sub> O <sub>3</sub> mg.	DECREASE %
Untreated	0.30	-	0.37	-	0.32	-
H <sub>2</sub> O	0.16	47	0.23	38		
HCl (0.1%)	0.11	64				
NaOH (0.1%)	0.09	70	0.044	87	0.09	72
Na <sub>2</sub> CO <sub>3</sub> (0.2%)	0.08	74	0.03	87		
Ether					0.06	82
Ethanol					0.04	88

TABLE II  
EFFECT OF ALCOHOLIC AND AQUEOUS EXTRACTION AT 20° AND 100°

SOLVENTS	EXTRACTION AT 20°		EXTRACTION AT 100°	
	As <sub>2</sub> O <sub>3</sub> mg./100 Gm.	DEGREE OF EXTRACTION %	As <sub>2</sub> O <sub>3</sub> mg./100 Gm.	DEGREE OF EXTRACTION %
Untreated	1.60	-	1.60	-
Alcohol	1.23	23	1.23	23
Alcohol + HCl	1.10	31	0.93	44
Alcohol + NaOH	0.64	60	0.23	86
Alcohol + HCl + NaOH	0.29	82	0.13	92

TABLE III  
CONCENTRATION OF As<sub>2</sub>O<sub>3</sub> IN NORMAL HAIR BEFORE AND AFTER EXTRACTION

SAMPLE		As <sub>2</sub> O <sub>3</sub> (MG/100 GM.)	
NO.	WEIGHT Gm.	INITIAL	FINAL
1	3.95	0.33	0.031
2	4.39	0.12	0.032
3	3.67	0.09	0.023
4	4.64	0.24	0.033
5	3.32	0.48	0.041
6	5.87	0.13	0.025

*Experiments With Guinea Pigs.*—To test this hypothesis, it was necessary to try the method of treatment on hair of known arsenic content established by internal deposition. Four guinea pigs were treated such that two were injected daily subcutaneously with 0.75 c.c. of a sodium arsenite solution (1 mg.

$\text{As}_2\text{O}_3$  per c.c.), and two served as controls. The first animal died after receiving 6.5 mg.  $\text{As}_2\text{O}_3$ , and the second after 9.5 mg. About 15 Gm. of hair were clipped from each animal. Approximately 3 Gm. were used in each determination, extracting for eight hours with 95 per cent ethanol and refluxing in a steam bath for two hours each with 0.1 per cent NaOH and 0.1 per cent HCl. The results are given in Table IV.

This result indicates that arsenic appearing in hair from the systemic circulation is not irreversibly fixed and is capable of being extracted in the course of removing arsenic acquired by external contamination. Using the less drastic procedure of extracting with 0.5 per cent  $\text{Na}_2\text{CO}_3$  in place of 0.1 per cent NaOH gave essentially the same result. An analysis of the fluids obtained in the extraction of the hair from guinea pig 4 showed that the alcohol had removed 11 per cent, the NaOH 75 per cent, the HCl 7 per cent, and 7 per cent remained in the hair.

TABLE IV

EXTRACTION OF GUINEA PIG HAIR AFTER SUBCUTANEOUS ADMINISTRATION OF ARSENIC

ANIMAL	$\text{As}_2\text{O}_3$ CONTENT		EXTRACTION
	UNTREATED	TREATED	
	mg.	mg.	per cent
1 Control	0.03	0.03	0
2 Control	0.03	0.03	0
3 Injected	1.06	0.07	94
4 Injected	1.30	0.10	92

One of the control guinea pigs was next injected on two successive days with 0.75 mg. of  $\text{As}_2\text{O}_3$  as sodium arsenite and then clipped. The value of arsenic in the hair rose from 0.03 to 0.1 mg. per 100 Gm. We consider this a significant experiment in that it demonstrates the very rapid appearance of arsenic in hair from a systemic origin. It could not possibly reach the hair shaft by fixation as an insoluble keratin complex laid down in the follicle. It must have reached its location by secretion from sebaceous or sweat glands and by adsorption or absorption from fluid bathing the shaft. We have repeated this experiment on two additional guinea pigs determining the arsenic content of the hair on the same animal both before and after injecting the arsenite. The arsenic content rose from 0.11 to 0.16 mg. per 100 Gm. of hair. It is thus necessary to conceive of two sources of arsenic in hair: (1) a small percentage as fixed in the follicle and appearing in the shaft with growth, and (2) a large percentage appearing on or in the shaft from secretions of sweat or sebaceous glands. Our evidence does not at present permit differentiation of these sources.

*Adsorption Experiments.*—The problem was next approached by attempting to determine the extent of the removal by various solvents of arsenic applied externally to hair. Smith and Hendry<sup>11</sup> have claimed that only 40 per cent of such arsenic could be removed after soaking in water for fifteen days.

#### *Adsorption and Elution of Sodium Arsenite.*—

*Experiment 1.*—13.8 Gm. of hair were extracted for five hours with alcohol in a Soxhlet apparatus. It was then placed in 200 c.c. sodium arsenite, equivalent to 0.002 per cent  $\text{As}_2\text{O}_3$ . One c.c. or more of the solution was removed at

intervals, and the arsenic concentration was determined. The results are shown in Fig. 1. The hair extracted all of the arsenic in seventeen days. The pH of the solution changed from 8.5 to 5.4. The initial concentration in this specimen of hair was 0.034 mg.  $\text{As}_2\text{O}_3$ /100 Gm., and the final 29.0 mg.

In an attempt to remove this "adsorbed" arsenic, the specimen of hair was soaked in 900 c.c. of water for twenty-four hours, filtered on a Buchner funnel, and washed with 100 c.c. of water. The combined filtrates were concentrated to 100 c.c., and the arsenic concentration was determined. This showed a loss of only 7 per cent of its arsenic content

The specimen was divided into two portions, A and B. A (6.4 Gm.) was placed in 200 c.c. of water at pH 5.9 and soaked for twelve days with periodic determinations of the arsenic content of the medium. The  $\text{As}_2\text{O}_3$  content of the hair fell from 0.29 mg./Gm. to 0.10 mg./Gm. in eight days and remained constant thereafter. This represents a loss of 66 per cent for this operation. Refluxing with 1 per cent formic acid in ethanol for fifteen hours removed an additional 2 per cent.

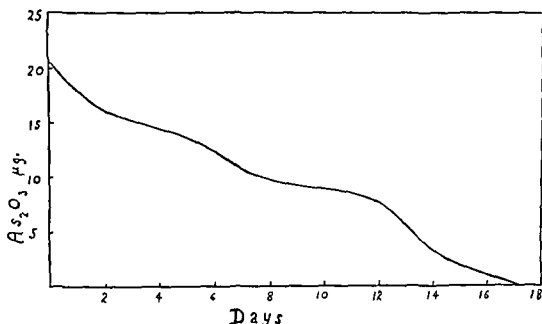


Fig. 1.—Rate of removal of arsenic from a solution of sodium arsenite (20  $\mu\text{g}$  per 100 c.c.) by human hair.

B (6.1 Gm.) was allowed to remain in water for sixteen hours; this decreased the  $\text{As}_2\text{O}_3$  content from 0.27 mg./Gm. to 0.23 mg./Gm. of hair. The specimen was then divided into two portions, B<sup>1</sup> and B<sup>2</sup> and extracted as shown in Table V. The results are expressed as the percentage decrease of the initial value for the particular procedure used. Alcoholic acetic acid (1 per cent) and aqueous hydrogen peroxide (3 per cent) were the only effective reagents.

*Experiment 2.*—After alcoholic extraction, another specimen of hair weighing 13.94 Gm. was soaked in sodium arsenite solution containing the equivalent of 1.0 mg.  $\text{As}_2\text{O}_3$  per 100 c.c. The hair contained originally 0.14 mg./100 Gm. and it absorbed arsenic from solution for fifteen days until the medium was 50 per cent of its initial concentration. This specimen was dried and used in the preparation of keratine.

Another alcohol-extracted sample of hair weighing 14.23 Gm. was placed in 250 c.c. of sodium arsenite solution containing 0.02 mg.  $\text{As}_2\text{O}_3$  per c.c. Most of the arsenic had been removed from solution in twenty days. The H ion con-

centration of the solution was kept between pH 5.3 and 5.6. When no further arsenic was removed from solution, NaOH was added to pH 11.8. A strong odor of hydrogen sulfide was perceptible, and the solution became colored. The concentration of arsenic returned to the initial level in two days.

*Experiment 3.*—14.58 Gm. of alcohol-extracted hair were placed in 200 c.c. of sodium arsenite buffered to pH 4.1 containing the equivalent of 3.4 mg.  $\text{As}_2\text{O}_3$ . At the end of five days there was no change in the concentration of arsenic.

*Separation of Cortex and Medulla.*—An attempt was made to separate cortex and medulla following the method of Jordan-Lloyd and Marriott<sup>12</sup> for goat hair to determine whether the arsenic was unevenly distributed in the hair shaft. Four N sodium hydroxide proved, however, to be too strong for human hair and there resulted a brown solution which left no residue on centrifugation. This was also true with a 3.3 N. No further attempts were made.

*Adsorption of Arsenic by Silk Fibroin.*—The adsorptive power of silk fibroin was next tested to determine whether a similar scleroprotein and a keratin would behave in an identical manner. There were placed 13.3 Gm. in 250 c.c. of sodium arsenite solution containing 0.02 mg.  $\text{As}_2\text{O}_3$ /c.c. There was no detectable decrease in the arsenic content of the solution after fourteen days. The initial concentration of arsenic in the fibroin was 0.23 mg.  $\text{As}_2\text{O}_3$ /100 Gm., and the final 0.26 mg.

TABLE V  
EXTRACTIONS OF FRACTIONS B<sup>1</sup> AND B<sup>2</sup>

FRACTION	TREATMENT	TOTAL $\text{As}_2\text{O}_3$ mg.	$\text{As}_2\text{O}_3$ mg./Gm.	DECREASE per cent
B <sup>1</sup>	Initial	0.470	0.232	
	NaOH (0.1 per cent) refluxing for two hr.	0.466	0.229	1
	HCl (0.1 per cent) refluxing for two hr.	0.458	0.227	1
	Alcoholic acetic acid (1 per cent)	0.058	0.029	88
B <sup>2</sup>	Initial	0.95	0.232	
	Alcohol, refluxing for four hr.	0.93	0.228	2
	Alcohol, refluxing for twenty hr.	0.90	0.220	3
	Alcoholic HCl (0.1 per cent)	0.89	0.218	1
	$\text{H}_2\text{O}_2$ (3 per cent) at 20° for twelve hr.	0.88	0.216	1
	$\text{H}_2\text{O}_2$ (3 per cent) refluxing for four hr.	0.76	0.187	14
	NaOH (0.1 per cent) refluxing for one hr.	0.74	0.182	3
	HCl (0.1 per cent) refluxing for one hr.	0.71	0.174	5
	Alcoholic formic acid (1 per cent)	0.70	0.172	1

*Adsorption of Arsenic on Keratine.*—The hair remaining from Experiment 2 with adsorbed arsenic was treated after the manner of Goddard and Michaelis<sup>13</sup> with a solution of sodium thioglycolate (0.5M) of pH 11.4. When the hair had dissolved completely, the keratine was precipitated by acidifying to pH 6. It was centrifuged; the precipitate was redissolved in 0.1 per cent sodium carbonate and reprecipitated. This was repeated three times. The material was then dried, ashed, and the arsenic content was determined. Another specimen of normal hair which had not been exposed to arsenic in solution was also converted to keratine and analysed. The results were as follows: normal hair, 0.10 mg. per 100 Gm.; keratine derivative, 0.086 mg.; hair exposed to arsenite, 0.13 mg.; keratine derivative, 0.015 mg. In the case of normal hair the arsenic followed the keratine derivate, but this was not true for the hair with adsorbed arsenic.

## DISCUSSION

Most investigators and analysts have been content to wash hair in water prior to analysis for its arsenic content and to assume this sufficient to remove any external arsenical contamination. Wührer<sup>1</sup> extended this treatment to several washings with boiling water and 96 per cent alcohol in the cold for the estimation of the normal arsenic in human hair. His values ranged from less than 0.005 up to 0.095 mg.  $As_2O_3$  per 100 Gm. hair. Most of his 30 specimens, however, were less than 0.038 mg., and this agrees astonishingly well with our values on specimens of extracted hair. The problem of differentiation between arsenic originating from a hematogenous source and that from external sources seems to have been appreciated only by Smith and Hendry.<sup>11</sup> These investigators have suggested tentatively that prolonged soaking in water would differentiate between arsenic absorbed *in vivo* and arsenic resulting from external contamination, or what we have called "internal" and "external" arsenic in this paper. On the other hand, they state that only 40 per cent of the arsenic absorbed from a solution of sodium arsenite by hair could be removed after fifteen days' soaking in water changed every day. The experiments given above, however, demonstrate that such arsenic can be removed to the extent of 66 per cent by water alone and even 74 per cent by other agents. This is unfortunately also true in general for arsenic originating *in vivo*.

The objection might be raised that the treatment of two hours in boiling 0.1 per cent NaOH or HCl was too drastic. It must be realized, however, that despite this treatment some arsenic remains fixed in the hair, whether originating "internally" or "externally." It is possible that the isoelectric point of keratin at pH 4.9 plays a role in this phenomenon and that the absorption is chemical as well as physical. The fact that fibroin is inert and differs chemically from hair keratin as regards its sulfhydryl and disulfide groupings is also suggestive of chemical "fixation" of arsenic. This explanation is also suggested on the basis of the kerateine experiments.

We are therefore forced to the conclusion that it is not possible to differentiate between arsenic originating internally from that of external origin. High values will still be of importance from a medicolegal point of view, but low values require definite evidence of absence of exposure to external sources. Our guinea pig experiments would suggest that to retain hematogenous arsenic, soaking in water for a few hours and washing with alcohol are all that are permissible. This procedure, however, will not remove arsenic from external contamination.

## SUMMARY

Tests have been carried out to determine the effect of treating human hair prior to estimating its arsenic content in medicolegal practice. Ethanol or ether followed by dilute hydrochloric acid and sodium hydroxide removed 70 to 90 per cent of the arsenic present. This procedure, however, also extracted over 90 per cent in guinea pig hair after parenteral administration.

Hair soaked in sodium arsenite solution absorbed arsenic slowly over several weeks. This arsenic was removable to the extent of 66 per cent by distilled water. An additional 8 per cent could be extracted only with great difficulty

centration of the solution was kept between pH 5.3 and 5.6. When no further arsenic was removed from solution, NaOH was added to pH 11.8. A strong odor of hydrogen sulfide was perceptible, and the solution became colored. The concentration of arsenic returned to the initial level in two days.

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## DETERMINATION OF AMMONIA IN THE URINE\*

ANTHONY A. ALBANESE, PH.D., BALTIMORE, MD.

EFFORTS to eliminate some of the inconvenience prevailing in the determination of ammonia by the Van Slyke aeration technique<sup>1</sup> led to the construction of an apparatus of new design, the description and operation of which are presented here. Briefly, the novel features consist of a permanently connected aeration and receiving chamber so assembled that the alkali solution is admitted into a closed system which obviates the possible loss of ammonia inherent to the addition of solid alkali into an open system.

*Apparatus and Solutions.*—The apparatus (Fig. 1) consists of the following stock units: One 500 c.c. Drechsel gas washing bottle (*E*); one 300 c.c. cylindrical open top separatory funnel (*A*); one single bulb straight calcium chloride tube, 200 mm. long (*B*); one drain tube marked at 10 c.c. intervals (*C*); two outlet aeration bulbs; two hose cock clamps; several glass bends made of 6 mm. tubing. These various items are suitably mounted on a ring stand and assembled with alkali-boiled rubber tubing and stoppers.

*Solutions.*—The following are required: 0.05 N hydrochloric acid containing methyl red, 0.05 N sodium hydroxide, 0.0125 N ammonium sulfate, 80 per cent potassium carbonate, 10 per cent castor oil in 95 per cent ethanol.

*Procedure.*—The stopper of receiving chamber *A* is lifted and 10 c.c. 0.05 N HCl are added from a pipette. The volume of solution is increased to the 50 c.c. mark with distilled water. Now, the stopper of aeration chamber *B* is raised and distilled water is added to mark 1; 5 c.c. of urine or a suitable volume of any sample containing 0.3 to 2.0 mg. ammonia N is pipetted into this chamber; distilled water is added to mark 2, and finally a few drops of castor oil solution are added to inhibit excessive foaming. Both stoppers are made secure, and 10 c.c. of 80 per cent  $K_2CO_3$  are run into *B* from alkali reservoir *C* by manipulation of hose cock Clamp *I*. The trapped suction is turned at half optimal speed for two minutes and at optimal speed for eight minutes. The optimal speed is determined by the aeration rate which can be maintained without entraining fluids into the outlet tubes. This is prevented to a great extent by the use of U-tube endings for the aeration outlets as shown in the diagram. The duration of aeration necessarily varies with aspirators and should be determined by running a check with 5 c.c. of 0.0125 N  $(NH_4)_2SO_4$  (0.875 mg. N). When the requisite aeration period has elapsed, the suction trap is opened, the aspirator turned off, and stoppers to chambers *A* and *B* are loosened. The contents and washings of *A* are run into the 125 c.c. Erlenmeyer flask *D* and the amount of ammonia collected is determined by back titration with 0.05 N NaOH. The

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residue solution in B is discarded to the waste by releasing hose cock Clamp II. Both chambers are rinsed out thoroughly before the next determination.

*Performance Tests.*—A. The apparatus and method described below have been in use in this laboratory for more than two years, during which time more than 1500 ammonia determinations have been done. It has been found that

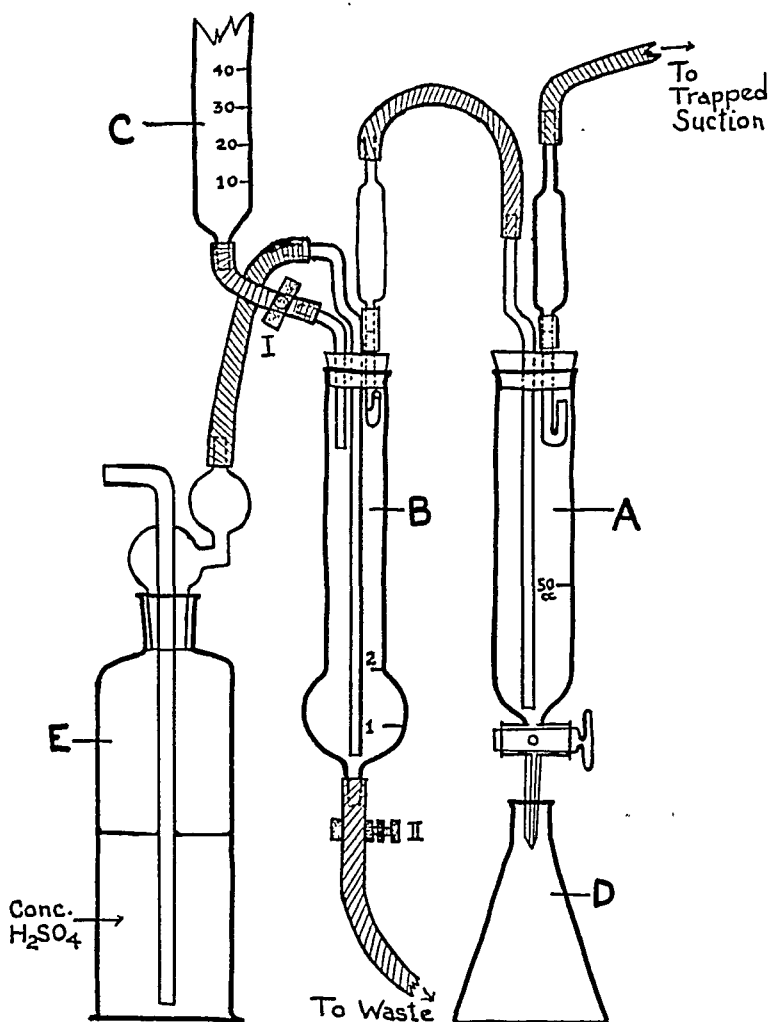


Fig. 1.

TABLE I

RECOVERY OF AMMONIA-N FROM THE ADDITION OF AMMONIUM SULFATE AND UREA TO URINE

SAMPLE	NITROGEN ADDED MG.	AMMONIA-N FOUND MG.	AMMONIA-N RECOVERED MG.	AMMONIA-N RECOVERED PER CENT
5 c.c. urine	0	1.33	0	0
5 c.c. urine + $(\text{NH}_4)_2\text{SO}_4$	0.69	1.99	0.66	95.8
5 c.c. urine + $(\text{NH}_4)_2\text{SO}_4$	1.390	2.73	1.40	100.1
5 c.c. urine + urea	4.50	1.34	0.01	0.22
5 c.c. urine + urea	13.50	1.36	0.03	0.22



duplicate determinations check easily within 0.1 per cent. The results of tests shown in Table I indicate the recovery of added ammonia-N to be quantitative and the interference of urea to be negligible.

*Summary.*—A new apparatus for the determination of ammonia has been described. It offers great ease of operation and accuracy.

#### REFERENCE

1. Van Slyke, D. D., and Cullen, G. E.: A Permanent Preparation of Urease, and Its Use in the Determination of Urea, *J. Biol. Chem.* 19: 211, 1914.

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#### Corrections

In the article "Permanent Metachromatic Staining of Gastric Mucus Smears" by Manfred Hess and Franklin Hollander in the March issue of this JOURNAL 29: 321, 1944, page 322, line 22 should read: "through two changes of 1 per cent HCl (1 c.c. of HCl, specific gravity 1.18, to 99 c.c. of distilled water)."

The use of 9 c.c. of water will yield a 10 per cent solution of HCl, which completely nullifies the metachromatic effect.

In the article "Studies on a Polysaccharide From the Tubercle Bacillus" appearing in the March issue of the JOURNAL, page 233, Table I, the route of injection should be I.Q. and not I.V. On page 234, in Table II, the average time of appearance in minimal cases is 3.8, not 38 hours.

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It is with deep sorrow that we have just learned of the sudden death of Dr. Warren T. Vaughan, Richmond, Va., on April 2. Dr. Vaughan served as Editor of this JOURNAL since October, 1923. An extended obituary will appear in a later issue.

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## BOOK NOTICES

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### Biochemistry for Medical Students\*

THIS new edition of Thorpe's *Biochemistry for Medical Students* warrants careful consideration by teachers of biochemistry in medical, dental and pharmacy schools. The order of presentation of the various subjects appeals greatly to the reviewer. For example, only a short chapter is devoted to digestion in general, leaving the specific digestion of the various energy foodstuffs to be discussed in the chapters dealing with their utilization. In these chapters digestion, absorption and intermediary metabolism are discussed in sequence. The chapter on lipids does not present as much of the recent conceptions of fat metabolism as would seem desirable. This is especially true of the section dealing with ketogenesis. A chapter on the utilization of oxygen and excretion of carbon dioxide has been added in this edition. The text on the whole is excellent.

J. C. FORBES

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### The Pharmacology of the Opium Alkaloids†

THIS is truly a monumental work covering every phase of the pharmacology of this most important group of compounds in detail. In the foreword by the late Doctor C. W. Edmonds is presented the historical background in connection with the development of this review. The bibliography contains more than 9,000 references beginning with the date of 1800 and going through 1942. These two volumes will be of much interest to every member of the medical profession; they are "must" books for pharmacologists.

H. B. HALL

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\**Biochemistry for Medical Students*. By William Veale Thorpe, M.A. (Cantab.), Ph.D. (London). Reader in Chemical Physiology, University of Birmingham. Third Edition with 39 illustrations, 476 pages, \$4.50. The Williams and Wilkins Company, Baltimore, 1943.

†*The Pharmacology of the Opium Alkaloids*. By Hugo Krueger, Nathan B. Eddy, and Margaret Sumwalt. Supplement No. 165 to the Public Health Reports, \$1.50 each. Part 1, 891 pages; part 2, 775 pages. United States Government Printing Office, Washington, 1941 and 1943.





Warren Taylor Vaughan  
1893-1944

# The Journal of Laboratory and Clinical Medicine

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Warren Taylor Vaughan

1893-1944

ALL too soon, death came, as it does to all men, great and small, to Warren Taylor Vaughan on April 2, 1944, in the early morning. Death struck with lightning swiftness and halted the dictations of thoughts from one of the keenest contemporary minds and one of the most prolific pens of the past two decades. Warren Vaughan had earned for himself the rare title of "The famous son of a famous father." He was the youngest of the five sons of Victor C. Vaughan, dean of the University of Michigan Department of Medicine and Surgery for thirty years, founder and editor of THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE. He followed in his father's footsteps and carried on in the editorship of this Journal in enviable style. He was one of the founders and an associate editor of *The Journal of Allergy*. He has contributed about two hundred articles to current medical literature.

Warren Vaughan's opus magnum, *Practice of Allergy* (1939), is outstanding in its field. His *Primer of Allergy* (1939, 1944) and a popular best seller, *Strange Malady*, both for the laymen, have been boons to sufferers. He was a pioneer internist allergist, worked untiringly for the application of the scientific principles of immunology, and wrote the early text on *Allergy and Applied Immunology* (1931). He has thus contributed immeasurably to the scientific development of this field. He was a founder, secretary general and treasurer (1928-1938), and in 1939 president of the American Association for the Study of Allergy. In the same year he held the presidency of the Society for the Study of Asthma and Allied Conditions. He was a member of the American Rheumatism Association, the Society of Investigative Dermatology, the International Society for Gastroenterology, and an honorary member of the Institute for the Practice of Medicine, Barcelona, Spain, and the Society for the Study of Allergy, Argentina. He served as a member of the councils of many nationally important organizations.

He received honors from various medical and honorary societies, and his Alma Mater bestowed upon him the honorary degree of Master of Science at

the Alumni Convocation on Founders' Day, October 4, 1941. At this time he delivered a very erudite and philosophic paper upon his unusual philosophy of life and his conceptions of duties and ideals. He was a crusader for the establishment and the maintenance of high ideals and high standards of practice and of research especially in his highly specialized field of medical practice. He was a leader, not only in allergy, but also in the broad field of internal medicine in which he was certified as a specialist by the American Board of Internal Medicine. In this Journal he wielded an influence for improved close relationships between laboratory and clinical medicine. He inspired his contributors in both experimental and clinical circles.

Warren Vaughan had an unusual ability for leadership that was evident even in his college days. In the medical school classes he was a great stimulus to all who had the privilege of knowing him. I was one of those fortunate enough to be close to him. His habits were exemplary. He encouraged the highest scholarship and accomplished the same for himself. In definitely unsolvable controversial matters, he took a sane middle-of-the-road attitude. He was an ardent supporter of whatever he deemed was right and was sharply critical of wrong, unethical, and dishonest actions. In his medical school days he was elected to the honorary fraternities of Alpha Omega Alpha and Sigma Xi. In his college days he was active in improving the standards of the fraternities. He was a member of Beta Theta Pi and Phi Rho Sigma of which he was author of the ritual and a councilor for many years.

Upon graduation from the University of Michigan in 1916 he served as house officer at the Peter Bent Brigham Hospital and won such distinction that ever afterward Michigan men had a definite advantage when they applied for service under "The Professor." Dr. Henry A. Christian was always his ideal of the master clinician. On completion of his house officership he accepted a position on the Harvard Medical School faculty in the Department of Public Health and Hygiene under Milton J. Rosenau. He took advantage of the abundant opportunities for the practice of preventive medicine in the field of internal medicine. When World War I was declared, he entered the Armed Forces and rose rapidly through all grades from the rank of first lieutenant to that of lieutenant colonel and chief of the medical service Camp Hospital 41 American Expeditionary Forces, Is-sur-tille, France. He returned to civilian life and practice in 1920 and chose Richmond, Virginia, as the place in which to do his lifework. He was director of the Vaughan-Graham Clinic. He contributed greatly to the high position of the practice of the art and especially of the science of medicine in that community.

Warren Vaughan assumed various editorial duties; in addition to his editorship of this Journal and associate editorship of *The Journal of Allergy*, he acted as collaborating editor of *Folia clinica chimica et microscopica* of Bologna, Italy. He was on the editorial board of *The American Journal of Digestive Diseases* and the *American Journal of Clinical Pathology*. He was a former member of the editorial board of *The American Journal of Syphilis, Gonorrhea, and Venereal Diseases*, and of *The Review of Gastroenterology*.

He was a member of the Committee on the Cost of Medical Care; a member of the Committees of Aerobiology and of Food Habits of the National Research Council; member since 1918 and, since 1938 on the council, of the American

Association for the Advancement of Science, member of the Research Council on Problems of Alcohol. He was active in the American Society of Clinical Pathologists, the Medical Society of Virginia (vice-president 1931-1932), Virginia Academy of Sciences, the Southern Medical Association, and the American Medical Association. He was a member of the Commonwealth Club.

In 1917 he married Emma Elizabeth Heath. To them were born four sons, Victor Clarence III, Warren Taylor, Jr., John Heath, and David DuPuy. His sons are carrying on the family tradition, the two older having received their medical degrees from Harvard Medical School and the two younger being students there.

Warren Vaughan will be greatly missed by every one of his many friends who have depended upon him for wise counsel. He died entirely too soon. We are prepared to accept as inevitable the passing of our older teachers, but we irreligiously protest the premature passing of our contemporaries just entering upon the promising harvest years of life. The abrupt premature closing of Warren Vaughan's scientific and medical career and his editorial work is bitterly lamented, but his personal charm, his inspiring interest in men and in science will long be remembered and will stimulate many to carry on in the high tradition that he forcefully exemplified.

—George Herrmann.

# CLINICAL AND EXPERIMENTAL

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## THE EFFECT OF CHEMOTHERAPEUTIC SULFA DRUGS UPON THE GROWTH OF $\alpha$ -HEMOLYTICUS STREPTOCOCCUS\*

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THE recovery of a few patients from subacute bacterial endocarditis when treated by combined fever and chemical therapies<sup>1</sup> suggested some investigations as to the rationale of this method of treatment. With this object in mind, attempts were made to determine whether the susceptibility of  $\alpha$ -hemolyticus *Streptococcus*, commonly found in this disease, could be modified in vitro by varying the conditions of cultivation, with special reference to the temperature of incubation. In order to study closely this relationship, it was deemed advisable to find the requirements for growth of the strain selected. Furthermore, a method<sup>2</sup> was developed to bring the drugs tested into continuous contact with the microorganisms by bubbling a suitable mixture of gases through the cultures during incubation. This method served also to assure a uniform temperature of the medium. By the use of various media it was possible to obtain growth curves, differing in the duration of the lag period, the phase of logarithmic growth, and the phase of decline. The results of the studies on the effect of sulfonamides on growth of  $\alpha$ -hemolyticus *Streptococcus* under various conditions are embodied in this paper.

### EXPERIMENTAL

A strain of  $\alpha$ -hemolyticus *Streptococcus*, obtained from the blood of a patient suffering from subacute bacterial endocarditis, is the microorganism used throughout these experiments. The strain failed to ferment esculin, was killed by exposure at 60° C. for half an hour, and was bile-insoluble. It was maintained by daily transplants in 1 per cent glucose broth incubated at 37.5° C. The cultures showed spontaneous agglutination forming granular deposits at the bottom and along the sides of the tube. After twenty-four hours of incubation, colonies appearing on the surface of "pour" plates of 1 per cent glucose agar and rabbit blood were discrete, small, slightly raised, and granular. In forty-eight hours the colonies became larger and more irregular. Biweekly serial transplants of supernatant of glucose-broth cultures into "buffer" broth were made, care being exercised to avoid transfer of the granular deposits. The "buffer" veal infusion broth contained 1 per cent peptone (Fairchild) and 0.2 per cent sodium phosphate at final pH 7.4. Thus, after two transplants, the

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colonies on agar plates appeared finely granular and the broth cultures uniformly turbid. The "smooth" characteristics of the cultures lasted for approximately four days; later a reversal to previous appearance was observed in all cultures.

# 1. STUDIES ON OPTIMUM REQUIREMENTS FOR CULTIVATION OF THE STRAIN OF $\alpha$ -HEMOLYTICUS STREPTOCOCCUS USED

The purpose of these experiments was to determine the effect of various factors upon the growth of the strain selected before proceeding with the main studies. The following is a short description of the findings:

A. Chart I illustrates the effect of different gas mixtures upon the growth of the strain of  $\alpha$ -hemolyticus *Streptococcus* in 1 per cent glucose broth at 37.5° C. and initial pH 7.6.

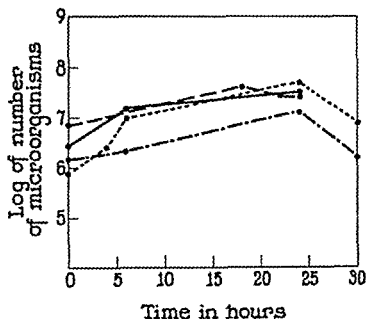


Chart I.—The effect of gas mixtures upon the growth of *Streptococcus viridans*.

..... 10% CO<sub>2</sub>  
 - - - - - 10% CO<sub>2</sub>, 5% O<sub>2</sub>, 85% N<sub>2</sub>  
 \_\_\_\_\_ 5% CO<sub>2</sub>, 95% O<sub>2</sub>  
 \_\_\_\_\_ 25% CO<sub>2</sub>, 75% O<sub>2</sub>

Incubation temperature 37.5 C. Medium 1 per cent glucose broth pH 7.6.

Chart I illustrates the growth curves obtained with various gas mixtures. The mixture consisting of 10 per cent CO<sub>2</sub>, 5 per cent O<sub>2</sub>, and 85 per cent N<sub>2</sub> produced a short lag period. The active logarithmic phase began in less than five hours following inoculation and continued for a period of twenty hours. This gas mixture was employed in all the experiments about to be reported in this paper. The apparatus previously described was used.<sup>2</sup> A series of culture tubes was always immersed at a depth where the level of the fluid in the tubes was below the level of the water in the water bath. Each tube containing 30 c.c. of 0.4 per cent glucose broth and seeded with 1 c.c. of an 18-hour broth culture of the microorganism was maintained in the water bath at the desired temperature. The mixture of gases was constantly bubbled through the media at a regulated speed, keeping the microorganisms in constant agitation and in immediate contact with the medium. The pH range used was 7.4 to 7.8. The bacterial counts were made by determining the number of colonies on "pour" plates of 1 per cent glucose agar and rabbit blood, seeded with 1 c.c. of the material tested at 6-, 24-, and 30-hour intervals.

B. In experiments described below,  $\alpha$ -hemolyticus *Streptococcus* was grown in various media incubated at different temperatures as indicated in Charts II and III.

One cubic centimeter of an 18-hour broth culture of the microorganism was seeded into each medium, and the gas mixture was bubbled through the media. Here again, samples were removed for bacterial counts at the beginning of the experiment and at 6-, 24-, and 30-hour intervals.

The results recorded in Charts II and III bring out the fact that the strain of  $\alpha$ -hemolyticus *Streptococcus* used may be cultivated at a wide temperature range. The higher the temperature, the shorter is the lag period. On the other hand, at 37.5° C. the logarithmic phase is of longer duration and the decline is slower than in the same media at higher temperature. The phase of decline roughly but consistently parallels the drop in the pH of the cultures. For this reason, the longest logarithmic phase is obtained in the best buffered medium containing rabbit serum and the most rapid decline in media containing glucose. Having completed the observations on the cultivation of  $\alpha$ -hemolyticus *Streptococcus* under various conditions, experiments were carried out on the effect of certain sulfa drugs upon the growth of this strain.

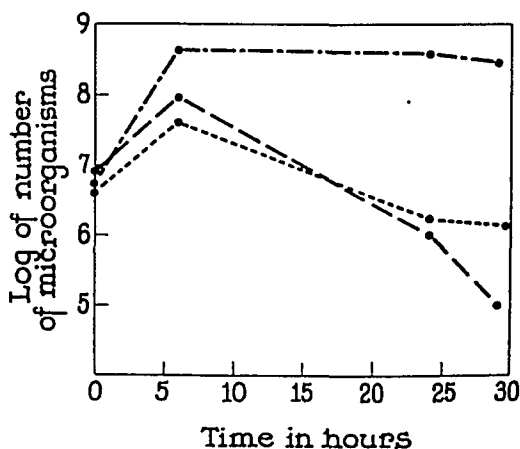


Chart II.—The effect of temperature upon the growth of *Streptococcus viridans*.

— 39° C  
 - - - 39.5° C  
 - . - . - 40° C

Gas mixture: 10 per cent CO<sub>2</sub> + 5 per cent O<sub>2</sub> + 8 per cent N<sub>2</sub>. Medium: 0.4 per cent glucose broth pH 7.4.

## 2. THE EFFECT OF SULFA DRUGS UPON THE GROWTH OF $\alpha$ -HEMOLYTICUS STREPTOCOCCUS

A. Various amounts of *sulfanilamide* were added to 30 c.c. of 0.4 per cent glucose broth of initial pH 7.4, thus giving final concentrations of 1:1500, 1:2000, 1:3000, and 1:6000. The tubes containing the mixtures were then seeded each with 1 c.c. of an 18-hour broth culture of the microorganism. The gas was bubbled through at a regulated speed. The temperatures tested were 39°, 39.5°, 40°, 40.5°, 41°, and 41.5° C. At the beginning of the experiment and after intervals of 6, 24, and 30 hours, samples were removed for bacterial counts.

It is seen from Chart IV that the various concentrations of the drug tested at various temperatures had no significant effect upon the growth of  $\alpha$ -hemolyticus Streptococcus. The growth curves were quite similar to the respective controls.

B. The effect of various sulfa compounds including sulfanilamide on the  $\alpha$ -hemolyticus Streptococcus was tested as follows:

*Series Number 1*

Tube 1, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum + 10 mg. sulfanilamide

Tube 2, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum + 10 mg. sodium sulfapyridine

Tube 3, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum + 10 mg. sodium sulfathiazole

Tube 4, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum (control)

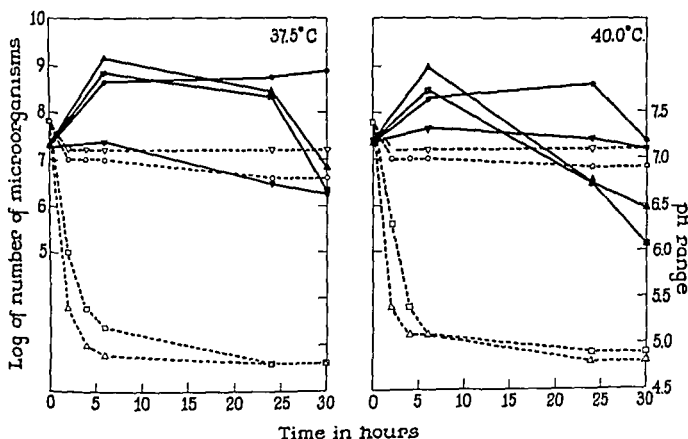


Chart III.—The effect of temperature upon the growth of *Streptococcus viridans* in various media.

—●— Plain broth—Rabbit Serum  
 —■— 1 per cent Glucose Broth  
 - - -○- - 1 per cent Glucose Broth—Rabbit Serum  
 - - -△- - Plain Broth

Gas mixture. 10 per cent  $\text{CO}_2$  + 5 per cent  $\text{O}_2$  + 85 per cent  $\text{N}_2$ .

*Series Number 2*

Tube 1, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum + 10 mg. sulfadiazine

Tube 2, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum + 10 mg. sulfaguanidine

Tube 3, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum + 10 mg. promin

Tube 4, 30 c.c. plain broth + 1.5 c.c. inactivated rabbit serum (control)

The final concentration of sulfa drugs in the media was 1:3150.

Each tube was seeded with 1 c.c. of an 18-hour broth culture of the microorganism. The initial pH was 7.4, the gas mixture was constantly bubbled through, and the temperatures tested were 37.5° and 40° C. Samples were removed for bacterial counts at the beginning of the experiment and after 6, 24-, and 30-hour intervals.

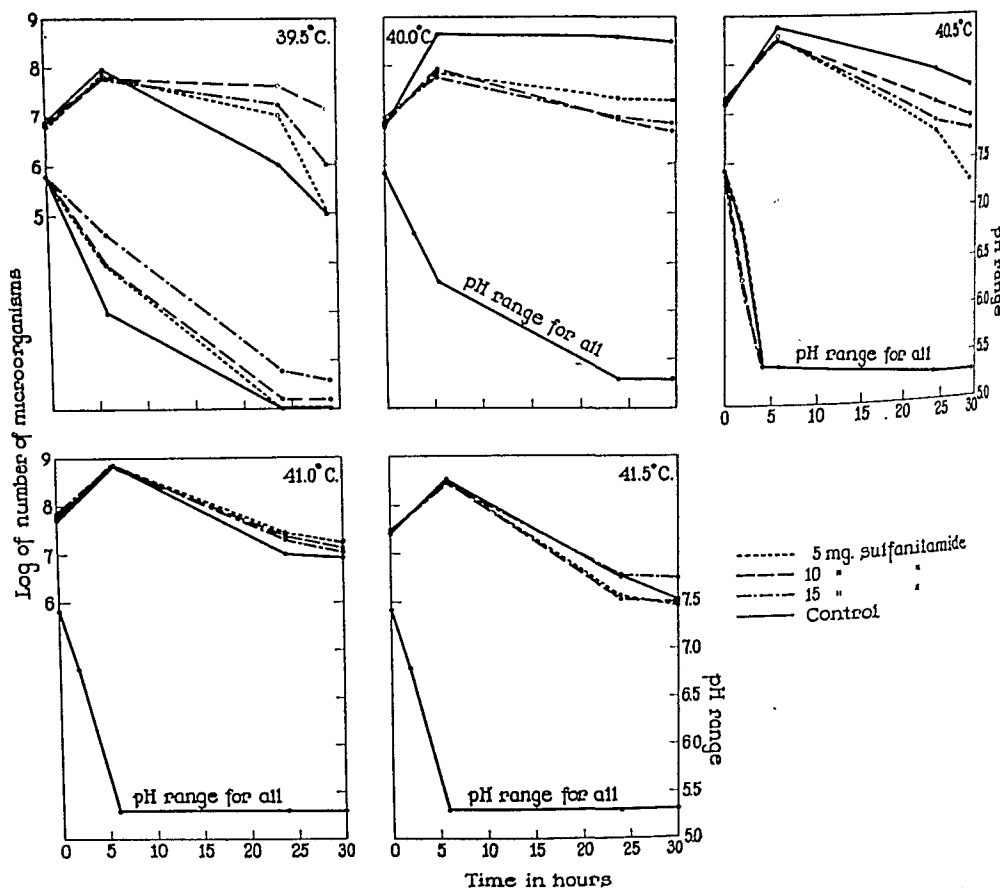


Chart IV.—The effect of various concentrations of sulfanilamide upon growth of *Streptococcus viridans* at various temperatures.  
Gas mixture: 10 per cent CO<sub>2</sub> + 5 per cent O<sub>2</sub> + N<sub>2</sub>. Medium: 0.4 per cent glucose broth pH 7.4.

Here again the sulfonamide compounds did not produce any appreciable effect upon the growth of the strain of  $\alpha$ -hemolytic *Streptococcus* tested. It may be seen from the examination of the counts of control cultures that various growth curves were obtained by the use of a wide temperature range and different media. The compounds tested failed, however, to inhibit the growth both under the least and the most suitable conditions of cultivation.

## COMMENT

In the work embodied in this paper, the effect of various sulfa compounds upon the growth of a strain of  $\alpha$ -hemolyticus *Streptococcus* isolated from the blood of a patient suffering from subacute bacterial endocarditis was determined by counting viable microorganisms in samples removed at frequent intervals from control and experimental cultures. The experimental setup used here was the same as previously described.<sup>2</sup> It permitted the frequent removal of samples without any interference with the growth and without change in the incubation temperature. It was accomplished by raising slightly the pressure of the gas mixture continuously bubbled through the culture and siphoning off the medium through a side tube. The gas mixtures used were selected after ascertaining their favorable effect upon growth. They were also responsible for continuous agitation of the cultures. Thus, the drugs tested were brought into constant contact with the microorganism. The agitation was considered important in the work in which  $\alpha$ -hemolyticus *Streptococcus*, having a strong tendency to form clumps and settle at the bottom of the fluid medium cultures, had to be employed. Incubation of cultures in a water bath with a sensitive thermoregulating mechanism and constant stirring<sup>3</sup> offered an opportunity for reliable and accurate temperature control which could be easily and quickly changed to any desired level. The different features of the method of cultivation described were used for various studies by previous authors singly and in combinations; to mention only the most recent studies of Longworth and MacInnes<sup>4</sup> on stabilization of pH in bacterial cultures; of Carpenter and Wingate<sup>5</sup> on cultivation of microorganisms at various temperatures; and of Magoon and Brunstetter<sup>6</sup> on cultivation of bacteria in constantly agitated media. It may be stated, however, that the combination of the various features in a somewhat simplified form served well the particular investigation described in this paper.

With special reference to the problem of this investigation, certain points should be emphasized.

The effects of sulfa compounds on  $\alpha$ -hemolyticus *Streptococcus* (*Streptococcus viridans*) received considerable attention of a number of previous investigators:

It seems that there exist considerable variations in the susceptibility of different strains to this group of drugs. According to Bliss, Long, and Feinstein,<sup>7</sup> most of the  $\alpha$ -hemolyticus *Streptococcus* strains have proved susceptible to 1:10,000 concentration of sulfanilamide. Some of the strains which were resistant were strains of *Streptococcus faecalis*, enterococcus being clearly resistant to the drugs (Neter<sup>8</sup>). According to Maegraith and Vollum,<sup>9</sup> the growth of  $\alpha$ -hemolyticus *Streptococcus* was affected by the drugs only in the presence of leucocytes. Bratton<sup>10</sup> investigated bacteriostatic effects of the drugs upon three freshly isolated strains of  $\alpha$ -hemolyticus *Streptococcus* and found no effect on two strains, while for the third strain sulfanilamide was bacteriostatic in a concentration of 1:1000 and bactericidal in a concentration of 1:500. Proseptasine had no effect; soluseptasine was bacteriostatic in 1.25 per cent and bac-

<sup>3</sup>Fisher Scientific Company, Constant Temperature Water Bath, Fisher Unitized Water Bath, Catalogue No. 15-444.

tericidal in 25 per cent; T 607 was bacteriostatic in a concentration of 1:4400; while T 626 was bacteriostatic in a concentration of 1:20,000 and bactericidal in a concentration of 1:10,000. Swain<sup>11</sup> determined the effect of 4:4'-diaminodiphenyl sulfone, sulfapyridine, and sulfanilamide upon four strains of  $\alpha$ -hemolytic *Streptococcus* isolated from cases of subacute bacterial endocarditis treated with the drugs. The two strains isolated from cases showing clinical improvement were susceptible, while the strains isolated from cases which failed to respond to the treatment were resistant to the drugs in vitro. Treadway<sup>12</sup> also reported that four strains of the  $\alpha$ -hemolytic *Streptococcus* showed wide variation in response to the drugs, sulfathiazole being the most effective.

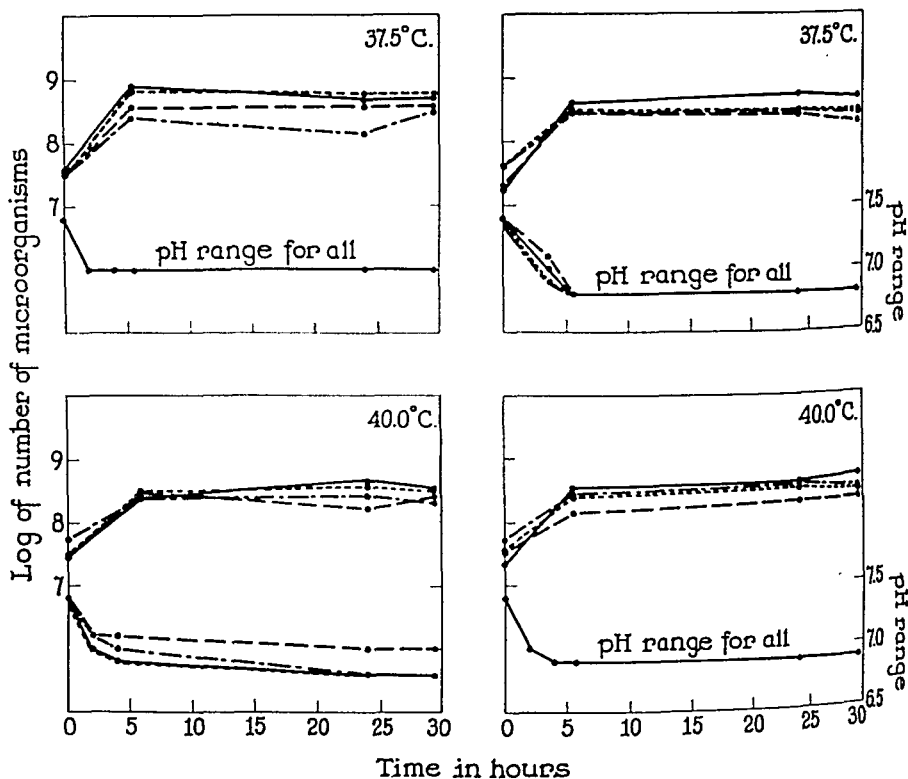


Chart V.—The effect of sulfanilamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfapromin, and sulfaguandine upon growth of  $\alpha$ -hemolytic *Streptococcus* at various temperatures.

Control  
Sulfadiazine  
Sulfapromin  
Sulfaguandine

Control  
Sulfanilamide  
Sulfathiazole  
Sulfapyridine

Gas Mixture: 10 per cent  $\text{CO}_2$  + 5 per cent  $\text{O}_2$  + 85 per cent  $\text{N}_2$ .

The cause of the variation in susceptibility of  $\alpha$ -hemolytic *Streptococcus* to the drugs in vitro still remains obscure. Gay, Clark, Street, and Miles<sup>13</sup> have reported that they were able to obtain killing of *Streptococcus* by sulfanilamide by 1:5000 concentrations in broth, when the cultures were incubated between 39 and 40° C. Bacteria grew more slowly and to a lower peak at 40° C. than at 37° C.

In the investigation reported in this paper a single strain, freshly isolated from a human case of subacute bacterial endocarditis, was studied. The strain proved at once resistant to the effect of drugs under the ordinary conditions of cultivation. A series of experiments was then carried out to determine whether this resistance could be changed under modified conditions of cultivation, i.e., various hydrogen-ion concentrations during the logarithmic and stationary phase of growth; the use of more and less favorable media; and finally the employment of various temperatures compatible with growth. In this manner, several types of growth curves were obtained; these differed in the duration and the level of the lag period, of the phase of logarithmic growth, and of the stationary stage. Under the conditions tested, there was only insignificant bacteriostatic, and no clear-cut bactericidal effect obtained by the use of sulfanilamide, sodium sulfathiazole, sodium sulfapyridine, sulfaguandine, sulfadiazine, and promin.

#### SUMMARY

The inherent resistance to sulfanilamide and various sulfa compounds of a strain of  $\alpha$ -Streptococcus hemolyticus isolated from a human case of subacute bacterial endocarditis remained unchanged under both favorable and adverse conditions of cultivation *in vitro*.

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# EFFECT OF CERTAIN SALTS ON ACTIVITY OF SULFACETAMIDE\*

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IT IS common knowledge that sodium citrate solutions are widely used in bladder irrigations after transurethral surgery because of their anticoagulant property. For obvious reasons, one of us (B. D. P.) wished to add sulfacetamide to this irrigating solution. Before clinical use, however, it seemed wise to investigate the bacteriologic behavior of the mixture inasmuch as sulfonamide derivatives are known to encounter "blocking" of their bacteriostatic activity in the presence of various agents, such as para-aminobenzoic acid, peptones, methionine, and sugar.

The first experiment with citrate and sulfacetamide produced such unexpected results that it was decided to employ two other salts for comparative purposes. Sodium acetate and sodium lactate were selected as representing anions which the drug must frequently meet in the body. However, stronger solutions than those present under physiologic conditions were used for study. Since the 4 per cent sodium citrate solution is the one employed in urologic irrigations, this strength was prepared and the other two salts were made of equimolar concentration.

## METHOD

Aqueous or dextrose broth solutions of each salt were prepared. The test sulfonamide was added to these in amounts equal to saturation in water. Aliquots (2.5 c.c.) were inoculated with 0.025 c.c. (0.1 c.c. of 1:4 dilution) of 18-hour dextrose broth cultures of the organisms. Control tubes were of two types: those containing solutions of the salts only, and those containing the sulfonamide solution alone. After 4- and 24-hour incubation periods, cultures were made for bacterial counts by plating in meat infusion agar to which para-aminobenzoic acid had been added. One-tenth c.c. and 0.1 c.c. of 1:100 dilution were used for plating. All solutions were tested for sterility.

## RESULTS

Sulfacetamide is regarded as an adequate bactericidal opponent of *Escherichia coli* and is frequently employed in urinary tract infections. In these experiments, recently isolated strains of *E. coli* were employed. All strains used gave typical reactions to the carbohydrates and were methyl red and indole positive. In the control tests using aqueous solutions of acetate, lactate, and citrate, the organisms grew without restraint, and innumerable colonies of *E. coli* were produced on all plates, both in the 4- and 24-hour cultures. Sulfacetamide in aqueous solution was found to give complete sterilization in cultures of three of the four strains tested after 24 hours' incubation. The fourth culture showed four colonies of *E. coli* in 0.1 c.c. after 24 hours. In tubes containing sulfacetamide with acetate, innumerable colonies of *E. coli* were produced in both the 4- and 24-hour platings. When the cultures were made omit-

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ting the para-aminobenzoic acid, inhibition of growth of the *E. coli* was demonstrated, denoting a bacteriostatic rather than a bactericidal action. The bactericidal potency of the drug did not seem to be impaired appreciably by the presence of sodium lactate, all cultures showing a striking diminution of growth in 24 hours. This was true when cultured both with and without the addition of para-aminobenzoic acid to the media. The same results could not be repeated when sodium citrate was added with sulfacetamide to the culture media; in this instance bacterial growth was unimpaired and innumerable colonies of *E. coli* were produced in 0.1 c.c. of 1:100 dilution in both the 4- and 24-hour cultures. The omission of para-aminobenzoic acid did not alter this finding. The interference of citrate with the antibacterial action of sulfacetamide was thus clearly demonstrated, and the drug was shown to be rendered inert in the presence of citrate.

For purposes of comparison, it was deemed advisable to establish the effect of these salts on other organisms. Therefore, freshly isolated strains of *Staphylococcus aureus* and *Streptococcus hemolyticus* were employed with the particular sulfonamide considered efficacious against that organism. Sulfathiazole was tested with staphylococci. The same method of preparing tubes for incubation was used with the single exception that meat infusion broth was employed and the appropriate sulfonamide dissolved in it.

Four recently isolated strains of *Staphylococcus aureus* were employed, and cultures were prepared in the method indicated. In the experiments where the test salts were acetate and lactate, the organisms survived unaltered. Even the addition of sulfathiazole failed to restrict perceptibly the colony count. When sulfathiazole alone was used, all the cultures disclosed innumerable colonies at 4 and 24 hours. A marked inhibition in bacterial growth was demonstrated, however, in the tests containing sodium citrate and the mixture of citrate and sulfathiazole. There was not a noteworthy difference between the control containing citrate alone and the mixture with the sulfonamide. Although the latter colony count was somewhat lower, it was not significantly so.

A similar result was obtained, using in the same manner three freshly isolated strains of hemolytic streptococci against sulfanilamide. Consistent observations were made, although the susceptibility of the various strains of streptococcus to the sulfanilamide was irregular. The results noted with the staphylococci were duplicated in these tests. The streptococci were inhibited by the solution of citrate in broth, and the inhibitory action was strikingly apparent in all studies in which mixtures of citrate and sulfanilamide were used. In one case complete sterilization was obtained. These preliminary studies indicate that sodium citrate, when added to culture media, inhibits the growth of staphylococcus and streptococcus organisms. Sodium acetate and sodium lactate display no conspicuous inhibition or acceleration of sulfonamide activity. The drug behaves with complete indifference to their presence.

#### SUMMARY

The presence of sodium citrate (4 per cent strength) in a medium containing sulfacetamide nullifies the antibacterial behavior of the sulfonamide and permits unarrested development of *E. coli*. The citrate alone does not inhibit the growth of these organisms. The bacteriostatic potency of the drug is not similarly impaired by sodium acetate or sodium lactate.

# AN INTRADERMAL TEST FOR VITAMIN C SUBNUTRITION\*

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## PRELIMINARY REPORT

THE test is based on the intradermal injection of a dye which will be decolorized by vitamin C. Slow decolorization of the dye would indicate that the tissues have an insufficient amount of vitamin C. Rapid decolorization would indicate a sufficient amount. A skin test is cheap, easy to perform, and quickly read. If it should reliably point out vitamin C subnutrition states, it would be a valuable addition to our armamentarium.

Up to now, the reported investigations have been on a method and technique suggested by Rotter<sup>1</sup> in 1937. He used 0.01 c.c. of a N/400 solution of dichlorophenol indophenol to raise a blue wheal of about 2 mm. in diameter. Rotter believed that if a wheal disappeared in less than 5 minutes, the tissues were saturated with vitamin C; that if the reduction time was from 5 to 10 minutes there was a normal amount of vitamin C in the tissues; and that there was a vitamin C deficiency if the reduction time was more than 10 minutes. A sharp division of opinion has been found in the subsequent reports of other investigators as to the efficacy of this particular test.

The following investigators have reported that this test was reliable. Portnoy and Wilkinson<sup>2</sup> found the method to be of value and thought there was a general correlation with blood vitamin C levels. Banerjee and Guha<sup>3</sup> found the intradermal test to be accurate for the assessment of the level of vitamin C nutrition in guinea pigs tested under controlled conditions. They also found good correlation between this skin test and the urinary excretion of vitamin C in nine healthy human subjects. Suzuki<sup>4</sup> reported that this intradermal test paralleled closely the blood vitamin C levels. Beck and Krieger<sup>5</sup> stated that this intradermal test and the saturation test for vitamin C corresponded closely. Slavich and Torrini<sup>6</sup> showed that the time required for decolorization in an individual was markedly decreased following intravenous injections of two to five hundred milligrams of vitamin C. Masuzawa<sup>7</sup> declared that this skin test was satisfactory in determining vitamin C deficiency. Reddy and Sastry<sup>8</sup> noted that the skin test corresponded well with dietary intakes and urinary excretion.

The following investigators have reported this intradermal test as described by Rotter to be entirely unreliable. Jetter,<sup>9</sup> Poncher and Stubenrauch,<sup>10</sup> Bakhsh et al.,<sup>11</sup> and Goldsmith et al.<sup>12</sup> reported independently that there was no correlation between the fasting blood ascorbic acid and this intradermal test. Gambigliani Zoccoli and Lombardo<sup>13, 14</sup> found this test unsatisfactory for diagnosis of vitamin C deficiency. Baraldi<sup>15</sup> and Cera<sup>16</sup> too concluded independently that this method was not sufficiently specific for its proposed use.

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Lima<sup>17</sup> found no correlation between this intradermal test and vitamin C saturation tests. Wright and MacLenathen<sup>18</sup> did not consider this intradermal test a reliable guide to the body vitamin C nutrition. They found the variations in the decoloration in the same patient too great to set up a normal range and that readings at different sites frequently varied considerably. Poulsen and Lieck<sup>19</sup> too found different results in the same patient in different arms and legs and did not feel the test was of practical value in the form advocated by Rotter.

The contradictory results reported by the various investigators of the Rotter test suggested that the test was not entirely satisfactory in that form. We planned to study the intradermal test in all its aspects. The study was not to be limited to the method described by Rotter. Different methods and techniques were to be investigated in an attempt to find one that would be a reliable guide to the body vitamin C nutrition.

#### ANIMAL EXPERIMENTS

The rabbit is able to synthesize its own vitamin C and keeps a fairly constant degree of vitamin C tissue saturation. The rabbit's abdomen, which has been shaved and cleaned, can be used for a large series of skin tests.

When as Rotter<sup>1</sup> suggested, 0.01 c.c. of a dichlorophenol indophenol solution was used to raise a wheal of about 2 mm., there were variations in the decolorization times among adjacent wheals. It was noted that the immediate area of needle puncture was a darker blue and took very much longer to decolorize than the rest of the wheal. This may be due to the destruction of vitamin C where the needle punctures the tissue, thus producing a lack of vitamin C at that point. With small wheals, this may have caused the variations. When larger wheals of about 4 mm. were raised, there was no longer any marked variation in the decolorization times, and the tests were easier to read. For example, five skin tests performed on Rabbit C at adjacent sites disappeared in times with a deviation from the average of less than 45 seconds. No attempt was made to regulate the amount of dye injected, but only the size of the wheal. In all subsequent animal work, wheals of about 4 mm. were used.

Different dilutions of dichlorophenol indophenol were used for the intradermal tests. The higher the dilution the shorter was the time required for decolorization. The more concentrated solutions required proportionately longer times for decolorization. (See Table I.) For example, in Rabbit M, an N/5000 solution was decolorized in 18 minutes while the N/500 took 12.8 minutes.

The intravenous administration of vitamin C reduced the time needed for decolorization in all rabbits. For example, in Rabbit R the intradermal test showed that a N/500 solution was decolorized in 9.8 minutes; after a 200 mg. intravenous injection the average time was 4.1 minutes. This shortened time was still present on the following day. It took six weeks for the decolorization time gradually to return to the preinjection time of 9.8 minutes.

#### RESULTS WITH HUMANS

When wheals of approximately 4 mm. were raised in the forearm, there was little variation in decolorization times in an individual. Five intradermal tests done at adjacent sites on the forearm in an individual disappeared in ap-

proximately the same time. The deviation from the average time was never more than 45 seconds. The 4 mm. wheal was used in all subsequent work.

Different dilutions of dichlorophenol indophenol were used for the intradermal tests. In an individual, the higher the dilution the shorter was the time proportionately required for decolorization. For example, in D.F., a N/300 solution was decolorized in 15.2, a N/600 in 8.4, a N/1000 in 6.3, and a N/1500 in 4.0 minutes. The problem was then to find a dilution with no toxic effect which would best differentiate in decolorization time between the various degrees of vitamin C tissue saturation. Preliminary skin tests in duplicate and triplicate with dilutions varying from N/1500 to N/350 were done on 167 patients. These were hospitalized children and adults. We knew approximately how much vitamin C was being ingested, and their vitamin C blood level was done at the same time as the skin tests.

TABLE I  
RELATIONSHIP OF SKIN TEST TIME TO THE DILUTION OF THE DYE

RABBIT	DILUTION OF DYE	SKIN TEST TIME—MINUTES		
		<i>I</i>	<i>II</i>	<i>Average</i>
L	N/500	9.6	9.8	9.7
	N/1000	5.0	4.0	4.5
	N/2500	2.8	2.9	2.9
	N/5000	1.2	1.6	1.4
M	N/500	12.9	12.9	12.9
	N/1000	6.9	5.9	6.4
	N/2500	3.5	3.4	3.5
	N/5000	1.9	1.8	1.9
O	N/500	8.8	9.8	9.3
	N/1000	4.0	4.5	4.3
	N/2500	2.8	2.8	2.8
	N/5000	1.5	1.7	1.6

It became clear that the most concentrated solution that could be used without toxic effect would best differentiate between tissue saturation and unsaturation of vitamin C. There are at least two reasons for this. First, the concentrated solution takes a proportionately long time to become decolorized so that interpretation becomes easier. Thus, a difference between 10 and 15 minutes of using a concentrated solution would compare with 1 and 1½ minutes of using a very dilute solution. Second, there are other reducing substances in the skin such as glutathione whose effect tends to be minimized, the more concentrated the solution of dye that is used. The results with the N/300 solution of dichlorophenol indophenol, which is the most concentrated solution that we have used extensively, will be reported in detail.

A N/300 solution is prepared by dissolving 24 mg. of sodium 2-6 dichlorophenol indophenol with about 35 c.c. of boiling distilled water in a 50 c.c. volumetric flask, cooling, and making up to volume. This solution is prepared freshly every other day.

The technique is essentially the same as that used in the Mantoux and Schick tests. An ordinary tuberculin syringe and a short No. 26 needle are used. The syringe is rinsed twice with the dye before the injection. A part of the forearm without hair or veins is cleansed with alcohol and then permitted to dry. About 0.05 c.c. is injected into the skin in order to raise a wheal of approximately 4 mm. (3.5 to 4.5 mm.). The exact time of injection is noted from a

stop watch. Any excess dye or drop of blood which may obscure the reading is wiped off. The time when the blue color has disappeared altogether is noted. Where the needle enters the skin there may be a pin-point darker blue spot. This is not to be considered in determining the decolorization time. (See photograph.)

This method and technique was used on children and adults in the hospital. These were patients picked at random for the test. Two hundred and eighty-five tests were done in duplicate on the same and opposite forearms, and a blood vitamin C determination was performed on the patient at the same time. The approximate amount of vitamin C in their diet during the days preceding the test was known.

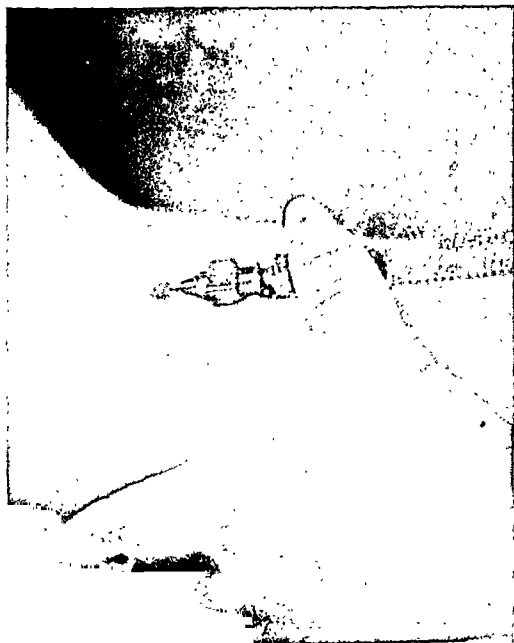


Fig. 1—Technique of the intradermal test.

One hundred and two patients had a skin test time of from  $3\frac{1}{2}$  to 9 minutes, 104 from 9 to 13 minutes, 79 from 14 minutes to 25.6 minutes. The blood levels of vitamin C ranged from 0.0 mg. to 1.97 mg.

There were 59 blood levels under 0.3 mg.; the mean was  $0.20 \pm 0.07$  mg. (See Table II.) The skin test time in 54 of these was 14 minutes or more; the other five were between 9.5 and 13.2 minutes; the mean was  $16.7 \pm 2.9$  minutes.

The method and technique which we have suggested and used obviates this criticism. Second, the skin test did not correspond to the blood vitamin C levels.

There has been a tendency to overestimate the value of the blood level of vitamin C in appraising nutritional status. Kruse<sup>21</sup> states "it should be clear that there is no necessarily high correlation between data derived by different methods on the same deficiency disease. They provide information on different aspects and states of the disorder. Unfortunately, this fact has not been appreciated. Rather, it has been thought that various methods applied to the same deficiency disease should yield similar data. On this basis it has become the practice to test the validity of a method by comparing its results with blood values. This procedure is entirely unsound. When it is remembered that blood values shift rapidly and may fluctuate intermittently, while tissue changes vary slowly, there should be no expectation of identical results. . . . Values on the concentration of a vitamin in the blood reflect very sensitively the recent dietary habit as well as other conditioning factors. They may change not only with season but also within shorter periods; they may fluctuate. . . . Potent therapy will produce maximum blood levels and entirely restore bodily saturation in several weeks, but will completely repair the slightest chronic tissue lesion only in months."

Our results in animals and human beings indicate that the skin test gives information as to the body saturation with vitamin C. The blood level reflects the recent intake of vitamin C. Therefore, the prolonged skin test time of over 14 minutes, which is usually associated with a blood level below 0.3 mg., and gross and biomicroscopic changes in the gums indicate a marked degree of tissue unsaturation. In some patients who took ascorbic acid on the day before the test the blood level was high, but the skin test remained prolonged over 14 minutes because the tissue reservoir was still depleted and had not been replenished.

At present we feel that skin test times of 9 to 13 minutes probably indicate mild degrees of unsaturation, while times of less than 9 minutes indicate satisfactory vitamin C nutrition. Skin test times of 7 minutes or less probably indicate complete saturation.

The blood level, skin test, gross and microscopic examination of the gums all give valuable information in evaluating the nutritional status as to vitamin C. The blood level indicates the recent intake. The skin test indicates the degree of body saturation and unsaturation. The gross and biomicroscopic examination of the gums indicate the changes that have taken place in the tissues. It must be remembered that once tissue changes have taken place, restoration to normal requires some time even though body saturation with vitamin C is maintained.

The skin test is so easy to perform, quick, and cheap that it may find a wide field of application. It can be used as one of the methods in evaluating the nutritional status as to vitamin C in an individual. It could be used in screening school children, hospital admissions, and the armed forces. The fact that the test can be read within twenty minutes makes it very convenient for office practice.

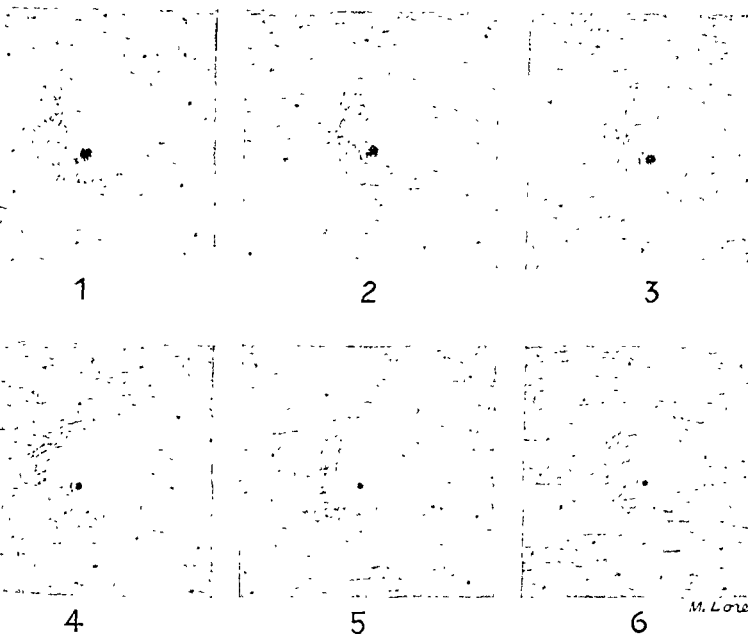


Fig. 2.—Intradermal test in patient with vitamin C subnutrition (painted from life).

1, Immediately after injection; 2, 4 minutes after injection; 3, 8 minutes after injection; 4, 12 minutes after injection; 5, 16 minutes after injection; and 6, 17½ minutes after injection, disappearance of all blue color except the pin-point darker blue spot where the needle entered the skin. This pin-point darker blue spot is not to be considered in determining the decolorization time.





The skin test would not take the place of gross and biomicroscopic examination of the gums. However, where gross and biomicroscopic examination have demonstrated tissue changes and the patient is being treated, the skin test could follow the efficacy of therapy. The best therapy is that which produces body saturation. Thus, the dosage of ascorbic acid should be that which will give a skin test time of seven minutes or less constantly.

It may also be possible to work out saturation tests with the skin test. At present the saturation tests are done by following the blood levels and urinary excretions of vitamin C after a test dose. These give valuable information but are time-consuming and difficult to perform. It seems likely that the study of skin test times before and after test doses may yield similar information with less effort and expense. The use of small test doses would be particularly helpful in those patients who had skin test times between nine and thirteen minutes and whom we consider to have mild degrees of unsaturation. When the skin test time is more than fourteen minutes, there is a definite vitamin C subnutrition.

This preliminary report is presented with the hope that others will check our results promptly. The intradermal test for vitamin C subnutrition can be useful during the war and postwar days.

#### SUMMARY AND CONCLUSIONS

1. Our intradermal test, raising an approximately 4 mm. wheal with a N/300 dichlorophenol indophenol solution, will indicate vitamin C subnutrition states.

2. Of 59 patients with blood vitamin C levels below 0.3 Mg., the skin test times were more than 14 minutes in 54. In 10 children on vitamin C deprivation diets, the blood levels fell, and the skin test times became prolonged. The skin test times were then reduced to normal following administration of ascorbic acid.

3. A skin test time of more than 14 minutes suggests a definite degree of body unsaturation, from 9 to 13 minutes mild unsaturation, and less than 9 minutes a normal amount of vitamin C in the body tissues.

4. Different tests give information on various aspects of the vitamin C nutritional state and thus will not always correlate. The blood level reflects only recent dietary intake. The skin test parallels the degree of body saturation. Gross and biomicroscopic changes in the gums demonstrate actual tissue changes.

I wish to thank Miss Joan Mestern for her valuable aid.

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## ON THE PERSISTENCE OF FALSELY POSITIVE SEROLOGIC TESTS FOR SYPHILIS IN NONSYPHILITIC INFECTIONS

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IN THE two decades following the introduction of the Wassermann test in 1904, false positive reactions were reported in a great variety of conditions, notably yaws, infectious mononucleosis, malaria, trypanosomiasis, relapsing fever, leprosy, tuberculosis, diabetes, pregnancy, anesthesia, malignancy, jaundice, fever in itself, and hypercholesterolemia. During the third decade after 1904, increasing refinement in technique, "notably in the direction of less anti-complementary antigens"<sup>9</sup> showed that of all these conditions only yaws gave a uniformly positive reaction, while mononucleosis, malaria, trypanosomiasis, relapsing fever, and leprosy did so occasionally, with a frequency that varied with different observers.<sup>8</sup> Both the Wassermann reaction and the more sensitive precipitation tests began to be considered as "amazingly specific," so that if these six diseases could be excluded, a positive, or certainly a repeatedly positive, Wassermann or Kahn test was taken as definitely indicative of the presence of syphilis.

Of late years it has become increasingly clear that the specificity of these tests was less than had been supposed. An interesting observation was made when the Wassermann and Kahn tests were applied to animals.<sup>6</sup> It was found that rabbit blood in particular but also that of chickens, pigeons, rabbits, mice, cattle, calves, sheep, lambs, horses, and swine frequently gave positive reactions. It is true that the reagin found in the blood of normal animals differs in certain fundamental respects from that of syphilitic blood:<sup>3</sup> it is more labile, is associated only with euglobulin instead of also with pseudoglobulin, and gives a complement fixation test with many lipoids that do not affect human syphilitic sera. In the routine tests, however, normal animal and human syphilitic blood behave exactly alike.<sup>5</sup>

The simplest method of distinguishing between the reagin of animal blood and that of syphilitic sera appears to be Kahn's verification test. Kahn<sup>5</sup> found that with his standard test human syphilitic serum gives a positive reaction at 37° C., but a negative one at 1° C. Animal blood does the reverse, being positive at 1° but negative at 37° C. Strongly positive blood sera of either kind may react at both temperatures; they must then be diluted.

The value of the Kahn verification test as a method of distinguishing between true and false serologic positive is still under discussion, some observers<sup>2, 12</sup> finding it quite trustworthy, while others<sup>10, 13</sup> reporting repeated failures. From the literature, one who has not used it gets the impression that while often useful, it is not always reliable.

Because of the presence in normal animal blood serum of a reagin that gives a positive Wassermann and Kahn test, it is not surprising that a similar

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reagin should have been found in normal human blood. Malloy and Kahn<sup>7</sup> in 1931, and after them a number of other observers,<sup>1, 14</sup> have shown that normal human sera contain this reagin, but ordinarily in too small an amount to show with our usual tests. Occasionally perhaps a normal human serum may contain enough of this reagin to give a positive test for syphilis. Eagle<sup>4</sup> in 1941 reported serologic studies done on 40,545 white college students of both sexes. In some ten cases (1:4000) a positive serology was found without other evidence of syphilis. Similar findings have been reported by others.<sup>14</sup> The interpretation of these findings is not easy. As Eagle points out, they may be:

1. True false biologic positive reactions
2. Cases after all of latent syphilis
3. Cases of recent intercurrent infection.

Unfortunately they could not be followed up serologically. Statistically the group of false biologic positive reactions is negligible, but in each individual case the interpretation of the reaction may be of the highest importance.

In cases of this kind, the possibility of error in laboratory technique cannot always be excluded. Since such technical lapses do occur, although rarely in the best serologic laboratories, no patient without other evidence of syphilis should be labelled syphilitic on the basis of serology alone until he has received the most careful kind of individual study. The following case may be in point: The patient, a married woman with healthy children, offered herself as a blood donor but was turned down because her Kahn test done twice was strongly positive on each occasion. Almost immediately her physician sent her blood to two other laboratories, by both of which Kahn, Kline, and Wassermann were reported negative. Was this a technical error? Perhaps not. O'Leary<sup>10</sup> speaks of a group of patients "who give biologic or false positive reactions which fluctuate from week to week and from month to month."

A much more important source of error is found in the nonsyphilitic infections that may show a positive serology. The general impression, however, is that these falsely positive Wassermann and precipitation tests are positive "only during the active stage of the disease."<sup>11</sup> That this is not the case I have had an opportunity repeatedly to note. The Kahn test may remain positive for as long as two weeks after defervescence as is illustrated by the following case:

Mrs. T. came to the office complaining of vague malaise, chiefly vertigo with headache, weakness, and a chilly feeling. Temperature, pulse, and respiration were normal, and physical examination was negative; in particular, there was no splenomegaly. The urine contained 3.5 per cent of sugar; the Kahn test was four plus. No blood spreads were made. A few days later a second tube of blood was sent to a private laboratory, and the Wassermann and Kline tests were each reported four plus. The patient and her husband were summoned to the office; the nature of syphilis was explained and prolonged treatment urged. Ten days later she returned to the office stating that she had had a chill two days before and again just before leaving home. Her temperature was found to be 105° F.; again no spleen was felt, but the blood contained many tertian parasites. There was prompt recovery under quinine therapy. Two weeks after defervescence, however, the Kahn test was still positive, though less intensely so than at first. After another two weeks, the test was negative and remained so. Interested by this case, Dr. Orgel and I

analyzed 154 cases of malaria, almost certainly nonsyphilitic, from the wards of the Jewish Hospital and City Hospital and from our own practice. The Kahn test was positive in 22 per cent, and in the cases in which a Wassermann was done, the same percentage of positives was found. What I should like to emphasize particularly, however, is the observation that of 13 cases followed after defervescence, 9 still showed a positive serology eight days after all evidence of fever had ceased, while one was still positive fourteen days after defervescence. All were negative fifteen days after defervescence.

A positive test for syphilis has been repeatedly found in rat-bite fever, but how long it may persist is shown by the following case.

M. L., aged 13, was brought to the Jewish Hospital on Oct. 14, 1934, with high fever and what looked like facial erysipelas. The mother stated that two weeks before admission the child had slept on the floor and had apparently been bitten under the left eye by a rat. The facial lesion had healed, but then had reopened with extensive cellulitis. After admission, she ran a high spiking fever for nearly two weeks. The temperature then became normal, but the lesion on the face did not entirely heal until a short time before her discharge six weeks later. Two mice received each 1 c.c. of the patient's blood intraperitoneally; after fourteen days, smears from one of the mice showed the presence of *Spirillum minus*.

A Kahn test was not done until two weeks after defervescence, when it was found four plus, the Wassermann being negative. The Kahn test was repeated at irregular intervals, thereafter, and was still four plus on Dec. 20, eleven days after she had been discharged clinically well. Unfortunately she then received in the Out-patient Department 5 injections of bismuth sodium tartrate and 3 of neosalvarsan. Following this, the intensity of the Kahn test gradually declined, becoming negative two months later. The Wassermann test remained uniformly negative.

It is of course possible that this little girl really had syphilis. There were, however, no history or physical signs of this disease, and in spite of the grossly insufficient treatment she has shown no evidence of syphilitic infection. She is now married and has given birth to a healthy child.

A more definite case of falsely positive serology was observed at the Jewish Hospital about a year ago.

M. P., aged 36, was admitted to the service of Dr. A. S. Reiches on Dec. 26, 1941, for a cellulitis of the nose following the picking of a nasal hair. The nose was red and swollen to the size of a golf ball. There was no fever, except that the rectal temperature once rose to 100°. Recovery was prompt under hot packs and sulfathiazole, and he was discharged well on Dec. 31. On admission, the Kahn test was found positive, the Wassermann negative; two days later the Kahn test was reported doubtful. In January, however, on two occasions, both Kahn and Wassermann were found definitely positive, only thereupon to become negative and to remain so spontaneously and without any treatment. At no time, then or later, did M. P. give any history of syphilis or show any signs suggestive of this disease. The serology may be summarized as follows:

DATE	KAHN	WASSERMANN	SPINAL FLUID
12/27/41	positive (J. H.)	neg. (J. H.)	
12/29/41	doubtful (J. H.)	insuff. (J. H.)	
12/31/41	discharged well		
1/19/42	+++ (Ives)	+++ (Ives)	
1/26/42	+++ (Ives)	++ Kolmer (Ives)	
2/ 2/42	neg. (Ives)	neg. (Ives)	
2/ 9/42	neg. (Ives)	neg. (Ives)	neg. (Ives)
4/ 7/42	neg. (Army board)	neg. (Army board)	
7/ 3/42	neg.	neg.	

Pneumonia has occasionally been found to give a falsely positive serology, and that this reaction may persist for some time after recovery is illustrated by the following case:

Mrs. F. S., aged 40, had long been troubled by chronic arthritis. She went to Excelsior Springs for the baths in 1937; a routine Kahn test taken there was found negative. She bore her first child early in 1941; Dr. Soule informs me that her Wassermann reaction then was negative. Her husband too, when qualifying as a blood donor, was found to have a negative Kahn test. I first saw her on June 2, 1942, for hypertrophic arthritis; a routine Kahn test then was negative.

On June 12, 1942, she entered the Jewish Hospital for an acute respiratory infection that rapidly proceeded to consolidation in the base of the right lung. There were no pneumococci in the sputum; there was a leucopenia and x-ray findings of confluent bronchopneumonia. The final diagnosis was pneumonia, acute interstitial, probably of virus origin. Defervescence took place on June 14, and she felt completely well thereafter. She was discharged on June 19. On the day of admission she showed a four plus Kahn, both Kahn and Wassermann remaining strongly positive for at least nine days after defervescence. She received no treatment, and since June her Kahn and Wassermann tests have been negative. The serology may be summarized as follows:

DATE	KAHN	WASSERMANN
6/12/42	++++ (J. H.)	
6/14/42	defervescence	
6/16/42	++++ (J. H.)	++++ (J. H.)
6/18/42	++++ (J. H.)	++++ (J. H.)
6/19/42	++++ (J. H.)	
discharged		
6/23/42	++++ (Hagebusch)	++ (Hagebusch)
8/24/42	neg. (J. H.)	neg. (J. H.)

The social and medical importance of these falsely positive reactions is much greater than the relative infrequency of their occurrence would indicate. The giving of a Kahn or similar serologic test has become more and more routine, not only in medical practice but in industry, before marriage, in connection with the draft, etc. Among such a huge number of tests many false positives must occur. Many of them are doubtless not recognized as such. The prevalent custom of considering every patient whose blood serum gives a repeatedly positive Kahn or Wassermann test as syphilitic and of immediately inaugurating treatment must mask many such cases. The serology promptly becomes negative and, at the close of a longer or shorter course of treatment, the physician concludes that he has cured his patient even though really the latter may not have been infected at all. A much greater disaster may result from the psychologic shock produced when a young man or woman is mistakenly convicted of being syphilitic, even though later the verdict may be withdrawn.

So far as the physician is concerned, the critical question is whether or not to inaugurate treatment in an individual who lacks history or signs of syphilis but who shows a repeatedly positive serology. It is today a commonplace that insufficient antisymphilitic treatment is worse than none and that one must choose between doing nothing and an adequate course of treatment that may take two years or more to complete. What then should be done with a patient who shows no other evidence of syphilis than a repeatedly positive Kahn or Wassermann or both? The following procedures will probably prevent error in most cases:

1. If the patient has recently had an acute infection of any kind, repeat the Wassermann and a precipitation test in a month or, better, two. If the test is still positive, the reaction is not due to the infection and is probably indicative of syphilis.

2. If a history of recent infection is not obtained, the patient may still be a member of the group described by O'Leary<sup>10</sup> who give "false positive reactions which fluctuate from week to week and from month to month." Here, weekly tests if not constantly positive may reveal the patient as not syphilitic.

3. If spinal puncture is done, its results may settle the matter, since a falsely positive spinal fluid has not as yet been reported.

4. In doubtful cases, Kahn's verification test may be employed, although as yet its results must be interpreted with reserve.

5. Sera that are positive with less sensitive techniques, such as the Wassermann test, but negative with more sensitive ones, such as the Kahn or Kline tests, are probably not syphilitic.<sup>8</sup>

#### SUMMARY

The occurrence of positive serologic tests for syphilis in the blood of non-syphilitic individuals is discussed. Its occurrence in various infections, not only during the active stage of the disease but for some days or weeks thereafter, is illustrated by four cases. A plea is made not to inaugurate treatment in a patient without other evidence of syphilis than a positive serology until further observation has made it clear that the reaction actually is due to syphilis.

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## DEATHS FOLLOWING THE USE OF LOCAL ANESTHETICS IN TRANSCRICOID THERAPY: A CRITICAL EVALUATION

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IT IS the purpose of this paper to emphasize the dangers associated with the use of cocaine and pontocaine when these agents are used in the preparation of patients for transcricoid therapy. A review of the literature on this subject would lead one to believe that fatalities following the use of these analgesics for pharyngeal anesthesia are by no means common. Our attention, however, has been directed to five deaths which have recently occurred in the Charity Hospital of New Orleans following the use of one or both of these drugs. Most of the patients were elderly and were being subjected to the instillation of iodized poppy seed oil for diagnostic purposes (Fig. 1). Of the five patients reported, four died before the instillation of the iodized oil, thus eliminating sensitivity to constituents of the oil, i.e., the iodine, the oil itself, or contaminants.<sup>1</sup> In the fifth instance, iodine sensitivity had been precluded by preliminary testing.

Detailed discussion of these cases serves little useful purpose because of their great similarity. An example which illustrates the sequence of events following the application of local anesthetics is that of a white woman, aged 65, who entered the hospital with chief complaint of pain in the neck and headache. The clinical impression at the time of admission was psychoneurosis, possible arteriosclerotic heart disease, possible Ménière's syndrome. During her stay in the hospital, a lipiodol instillation under the fluoroscope was suggested, to rule out bronchiectasis. Starting at 3:10 P.M., the patient's throat was sprayed with 5 per cent cocaine HCl, from a fine atomizer, at intervals of four to five minutes, for five times. She expectorated the excess cocaine. A total of about one cubic centimeter was used. The patient was talking during this time and showed no evidence of toxic reaction.

She walked across the hall to the fluoroscopic room. Under the fluoroscope, a catheter was inserted through the right nostril and into the trachea. The patient tolerated this well. About 4 c.c. of 2 per cent pontocaine were injected into the trachea. She coughed a little and tried to converse. A few seconds later, before the lipiodol was injected, she suddenly became rigid and slid off the stool onto the floor. She had a short clonic convulsion, became cyanotic, and expired. She did not respond to stimulants or to artificial respiration and was pronounced dead at 3:50 P.M. No gross or microscopic anatomical pathological findings to account for the death were noted. The relevant data of the other four cases are presented in Table I.

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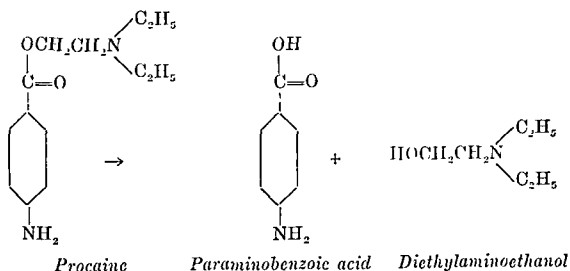


TABLE I

CASE	AGE	SEX	RACE	DRUG	SYMPTOMS	RESULTS	AUTOPSY	MODE OF DEATH
L. S.	65	F.	W.	Cocaine HCl, 5 per cent, 1 c.c. Pontocaine 2 per cent, 4 c.c.	Cough, inability to talk, short clonic convulsions, cyanosis	Death in 40 minutes	Negative	Respiratory failure
W. K.	56	M.	W.	Cocaine 5 per cent spray Pontocaine 2 per cent, several c.c.	Clonic convulsions	Death in 4 minutes	Negative	Respiratory failure
J. M.	72	M.	W.	Pontocaine 2 per cent, 15 c.c. spray	Cyanosis	Death in 6 minutes	Negative	Laryngeal asphyxiation
C. G.	51	F.	W.	Pontocaine 2 per cent, 2 c.c.	Difficult respiration, then apnea	Death in 15 minutes	Negative	Respiratory failure
E. B.	43	F.	C.	Cocaine, or Pontocaine	Frothing from mouth, restlessness	Death in 2 hours	Negative	Respiratory failure

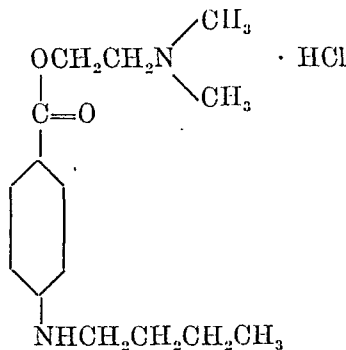
## DISCUSSION

Various local anesthetics are used today because they have the power of blocking locally the transmission of stimuli. Inasmuch as it is necessary to abolish swallowing and gag reflexes, the use of these drugs in transcrioid therapy is indispensable. A variable amount is absorbed depending to a large extent on the vascularity of the anesthetized area. Detoxification follows absorption. Goldberg, Koster, and Warshaw<sup>2</sup> have delineated certain of the steps involved in the detoxification of procaine. They found that procaine may be hydrolyzed at the ester link to form para-aminobenzoic acid and diethylaminoethanol, a reaction which takes place slowly in pure water and may be accelerated by catalysts.



Para-aminobenzoic acid has been extensively studied by a number of workers because of its intimate relationship with the sulfonamides. As in the case of these latter drugs, it is acetylated to form para-acetaminobenzoic acid. Many believe that the detoxification of these various substances takes place in the liver, but certain investigators<sup>2</sup> consider that at least a fraction is rendered inert in the blood stream. Goodman and Gilman<sup>3</sup> point out that the liver is capable of detoxifying one minimum lethal dose of cocaine in sixty min.

There are few detailed studies on the fate of pontocaine (parabutylamino-benzoyl dimethylamino-ethanol hydrochloride) whose base belongs to the procaine group.



*Pontocaine*

It is logical to assume, in view of this similarity, that a similar sequence occurs when it is detoxified.

Gilman<sup>4</sup> divides the reactions following the use of novocain into three main types: His first and most frequent is characterized by marked excitement, delirium and convulsions, and respiratory failure. This reaction begins within a variable period after administration of the drug and is frequently of long duration. His second type appears suddenly and, in contradistinction to the first type, is of short duration. In severe cases there is a pallor, fainting, and stoppage of the heart with resultant death. His third type of dangerous reaction is allergic shock. Waldbott<sup>5</sup> has reported urticaria, vasomotor rhinitis, and asthma from nupercaine, cocaine and similar anesthetics. Vaughan<sup>6</sup> has introduced a useful concept to indicate an increased sensitivity or reaction capacity to a foreign substance; namely, hyperergy. Hyperergic individuals react in a normal manner but react excessively. Vaughan illustrates this phenomenon by citing the person who sunburns extremely easily as being hyperergic to the actinic rays; another person who reacts with urticaria after similar exposure is allergic. It seems to us that the reactions following the administration of local anesthetics may be properly divided into two groups. Thus Gilman's first two groups are hyperergic; his first representing primarily nervous system involvement and his second representing primarily cardiovascular involvement.

With regard to Gilman's third or shock group, two possibilities exist. The first is that allergy is not a factor in the production of these reactions. It will be remembered that the allergic reaction is predicated upon the interaction of an antigen and the newly formed immune substance (probably globulin in nature) which has been synthesized stereochemically under the aegis of the sensitizing agent. By its very nature this process is time-consuming, requiring from five to seven days in experimental animals, and varying in the human being from this period to two years or longer.<sup>7, 8</sup> Since these reactions occur on the first exposure, no time has elapsed for antibody formation to occur. It must be emphasized that a latent period between the first exposure to an antigen and the following one must exist for an individual to react allergically. It must

also be borne in mind that the allergic reaction is usually initiated by a substance which is not primarily harmful; hence no ill effects are noted on the first administration.

The alternate hypothesis is that the individual has been sensitized by a previous exposure to a chemically related substance. Examples of naturally occurring substances related to these anesthetics would include the phenolic essential amino acids such as phenylalanine and tyrosine and para-aminobenzoic acid which is a component of the B group of vitamins. It is believed that the allergic reactions to drugs are mediated through linkage substances (Landsteiner) whose identity is unknown. It has been shown that many substances containing the benzene ring may be antigenic. Therefore the allergic hypothesis can only be satisfied if we assume a previous sensitization to a phenolic compound or even to para-aminobenzoic acid, a substance which is widely distributed throughout nature.

Thomas and Fenton<sup>9</sup> submitted samples of the pontocaine solution used in the three deaths they observed, to the Committee on Pharmacology and Chemistry of the American Medical Association. These were reported to contain unmodified, pure pontocaine and nothing else; it was concluded therefore that the fatal reactions were due to drug sensitivity and not to impurities.

Would it have been possible to have foretold sensitivity to pontocaine by the employment of appropriate measures? It is unfortunate that skin tests with drugs, which would be of great aid in helping avoid these reactions, are of questionable value. While patch tests to drugs causing contact dermatitis are highly specific and reliable, drugs which cause trouble following parenteral administration often cannot be satisfactorily tested either by patch test, scratch test, or intracutaneous test.

#### TREATMENT

The desideratum is for an anesthetic which combines long-acting and adequate anesthesia with no detrimental side effects. Up to the present, no drug meets all of these qualifications.

Furthermore, safe dosage levels have not been established for any of these agents. There is no such thing as a truly safe dose. A quantum can be established which can be tolerated by the vast majority of individuals, but no matter how small the dose used, there are persons who will succumb to it. This statement applies to all of these drugs, for Sollman<sup>10</sup> has indicated that in all probability all locally used anesthetics have produced fatal accidents.

In an attempt to attain the irreducible minimum of fatalities, the Local Anesthetic Committee of the American Medical Association<sup>11</sup> has made certain recommendations. They found that procaine was the safest of the local anesthetics and only if the concentration did not exceed one per cent. The total amount of cocaine should not exceed 0.06 to 0.1 Gm. (1 to 1½ gr.), but deaths have been reported from 0.02 Gm. (⅓ grain) of cocaine. It must be borne in mind that certain individuals have tolerated many times this amount. Of great importance is the site of administration. Fatalities are especially frequent when the urethra, scrotum, and tonsils are anesthetized.

Because it is known that the barbiturates have some slight antidotal effect, preoperative use of these sedatives is advisable. Fussgänger and Schaumann<sup>12</sup>

have shown that the addition of one drop of one-to-one-thousand epinephrine solution to 10 c.c. of 5 per cent pontocaine solution allows the operator to use twice the lethal dose. The epinephrine is added, not to protract the duration of anesthesia, but to decrease the rate of drug absorption. Provided the body's defenses are not immediately overwhelmed, the liver is capable of detoxifying one lethal dose of cocaine in an hour.

It is to be regretted that treatment of the severe reactions reported is of little value. The following procedures have been recommended, although admittedly little is to be gained from their use. Attempts to sustain heart action such as cardiac massage and intracardiac injection of epinephrine have been advocated. Artificial respiration rarely carries the patient through the crisis. When convulsions supervene, some sedative such as sodium amytal may be used.

#### SUMMARY

A series of five patients dying following the application of local anesthetics incidental to transdermoid therapy is reported. An attempt is made to explain the basis for these fatal reactions in the light of recent studies, and a rational classification of these reactions is presented. Prophylaxis and therapy are outlined.

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# THE ROLE OF CREATINE IN CELL GROWTH IN VITRO AND ITS USE IN WOUND HEALING

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## PRELIMINARY REPORT

CARREL<sup>1</sup> observed that extracts of muscle and gland tissues of adult animals stimulate the rate of growth of homologous fibroblasts in vitro. Carrel and Ebeling<sup>2</sup> demonstrated that chicken fibroblasts could be made to live and multiply indefinitely in vitro when the medium contained small amounts of embryonic tissue juice. They suggested that this proliferation is probably occasioned by certain nitrogenous compounds.

Recently Doljanski and co-workers<sup>3</sup> have extended these experiments to include the growth in tissue culture of human skin epithelium. They found a Tyrode extract of the heart muscle of various species not only strongly stimulated the growth of chicken cells but human skin epithelium as well, and they proposed the application of these extracts for wound healing.

The application of heterogenous extracts which contain various unidentified chemical entities, some inert substances, and possibly some inhibitors affords the scientific worker merely an empiric tool with which to initiate more fundamental studies. Indications of the wide field of application for tissue extracts make the identification of the substance or substances which manifest this cell activating and proliferating property one of paramount significance. The disclosure of an active substance will readily make available a definite class of substances for further study, will provide an opportunity to evaluate the specificity of a pure substance in relation to its cell growth-promoting and healing properties, and it may shed light upon other physiologic processes hitherto considered obscure.

This preliminary report is submitted to indicate that a successful attempt has been made to identify at least one of the active substances. A more comprehensive report will be detailed in the near future. Various tissue extracts, heart muscle extracts, and embryonic juice were found by chemical analysis to contain significant amounts of creatine and phosphagen.

Creatine was tested on the rate of growth of a variety of tissue cultures made through the courtesy of Miss Gladys Cameron in Professor Robert Chamber's Laboratory at Washington Square College, New York University. The results were found to be as follows: Tyrode solutions containing 400 to 800 mg. of creatine per 100 c.c. stimulate the growth of adult mouse bladder epithelium, adult mouse fibroblasts, human adult connective tissue and fibroblasts. Tyrode solutions containing as little as 100 to 200 mg. of creatine per 100 c.c. stimulate the growth of chick embryo fibroblasts, rat kidney and liver epithelium.

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A simple linear relationship exists between the creatine-induced area outgrowth of all cultures and the untreated tissue controls. This ratio is calculated by measuring the radii of the outgrowth areas. The mean radius of these measurements is compared with mean radius of the control. The areas represented by these radii will vary as the squares of their corresponding radii. The area ratios do not reveal the thickness and density of these outgrowths. In all the creatine-treated cultures the growth was thicker and more densely packed. In Table I are recorded a few typical results and the corresponding number of experiments performed to determine the activity of the creatine with respect to a specific tissue.

TABLE I  
THE EFFECT OF CREATINE UPON VARIOUS TISSUES

TISSUE	DAYS' GROWTH	NO. OF EXPERIMENTS	RATIO OF INDUCED OUTGROWTH AREA TO CONTROL AREA
Chick Fibroblasts	9	11	16:1
Mouse Bladder	6	16	6:1
Human Connective Tissue	10	10	4:1
Rat Liver	6	14	22:1
Rat Kidney	6	14	5:1

May we consider creatine and possibly other guanido amino acids as protoplasmic precursors? Clinical work is still in progress to determine the influence of the injections of solutions of various guanido amino acids upon the healing of internal ulcers. At present, these results are encouraging.

Sandweiss and co-workers<sup>4</sup> observed that pregnancy has a beneficial effect on the symptoms of peptic ulcer, and they focused their attention upon the anterior pituitary-like hormone.

Experimental ulcers were produced in dogs by the Mann-Williamson operation of surgical duodenal drainage. These Mann-Williamson dogs were given daily injections of varying doses of antuitrin-S. Because of this treatment 50 per cent had no ulcer and 20 per cent had ulcers in process of healing. Of the control Mann-Williamson dogs, 98 per cent died with jejunal ulcers. Patients suffering from active symptoms of peptic ulcer responded to antuitrin-S injections in a manner comparable to the Mann-Williamson dogs.

These experimental results provoked our interest in the possible effect of antuitrin-S upon growth of tissue cultures (in vitro). Accordingly, antuitrin-S powder\* was dissolved in Tyrode solution to yield a concentration of 400 I.U./c.c. The cell-proliferating property of this preparation was entirely negative for embryonic as well as for adult tissue. The tissues tested were human connective tissue, chick embryo fibroblasts, and epithelium and kidney tubules. From these results it is apparent that the beneficial effects of this hormone are not due to any direct effect upon ulcerated tissue, and we must search for an explanation of this healing mechanism.

Our disclosure of the value of creatine in cell proliferation and wound healing affords us a plausible explanation of the modus operandi of the anterior pituitary-like hormone.

\*Dr. Oliver Kamm of the Parke, Davis & Co. generously furnished us with the antuitrin-S powder.

It is known that the anterior pituitary hormones affect creatine metabolism, and the following three references selected from many others will tend to explain why pregnancy has a beneficial effect upon symptoms of peptic ulcer, and how injections of antuitrin-S may beneficially affect peptic ulcers.

Schrire and Zwarenstein<sup>5</sup> showed that during pregnancy the anterior pituitary gland hypertrophies increasing the formation of creatine. Gorostiaga<sup>6</sup> demonstrated that blood creatine during pregnancy and childbirth is increased 7.1 to 7.6 mg. per 100 c.c. Shapiro and Zwarenstein<sup>7</sup> studied the South African toad and noted 15 per cent decrease in muscle creatine following hypophysectomy. Daily injections of anterior lobe extract produced an increase of 30 per cent in muscle creatine.

Daily injections of anterior pituitary hormone increase the formation of creatine, its retention in various tissues, and its concentration in the blood. On the basis of our disclosure of activity of creatine as wound healer we conclude that the similar activity of anterior pituitary hormone in vivo is in part at least due to its effect upon creatine metabolism.

The author is indebted to Dr. Frances Krasnow for her cooperation in this study.

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## STUDIES IN COMBINED DIURETIC THERAPY\*

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### INTRODUCTION

IT IS well known that digitalis becomes finally ineffective in perhaps one-half the cases of congestive heart failure. It is least effective and most toxic in the most advanced forms. Since Jendrassik's introduction of calomel in the therapy of edemas, in Wagener's clinic in Budapest, from 1892 on, the search for more potent and relatively nontoxic diuretics has continued. Guy's hospital pill incorporated 1 grain each of calomel, digitalis, and squill. Merbaphen or novasurol, the first organic mercurial introduced by Saxl and Heilig in 1920, was too toxic and was soon superseded by mersalyl or salyrgan. At present we have the clinical choice of mercupurin, salyrgan-theophylline, esidrone, and some less well-known organic mercury compounds made in 10 and 13.5 per cent solution of the mercurial salt for intravenous or intramuscular use. Most of them now contain theophylline to the extent of some 5 per cent of the solution. The combination is more easily absorbed<sup>1</sup> and less irritating and destructive to the tissues. This may be due to the addition of the somewhat acidic theophylline to the corrosive alkaline hydrolytic products of the mercurials. Mercupurin was thus shown to be much less irritant locally than the original mersalyl by DeGraff and Batterman in 1935.

With the introduction of acid-forming salts, urea, hypertonic glucose, and the xanthine group as additional diuretics, the idea of combining them for additive and possibly synergistic effects arose. This was fostered by the elucidation of their varied modes of action, such as the production of urea and acidosis in the case of ammonium chloride and the direct renal effects of mercurials. Thus Keith and Whelan<sup>2</sup> found that ammonium chloride and novasurol produced greater diuresis than either alone, and together they were effective in cases that were otherwise refractory.

Since 1931, while Schmitz<sup>3</sup> was working with dogs, the Texas group<sup>4,5</sup> has studied the effects on patients in congestive failure with edema, attempting to prove the idea that the xanthines act primarily by increasing glomerular filtration, and the mercurials by blocking tubular reabsorption of fluid. Some have dissented.<sup>7</sup> Through the study of renal clearances of creatinine, d-xylose, urea, and more recently of diodrast and inulin, these ideas have been and are being put to the test. In an early paper<sup>4</sup> in 1933 from this Cardiovascular Service this statement was made: "These observations and facts suggested a possible complementary therapeutic combination of the two modes of action by a proper sequential or simultaneous administration of the two types of diuretics." Theophylline ethylenediamine was administered by mouth or in-

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travenously together with intravenous salyrgan, and increases of urinary output of 100 to 400 per cent were noted. In one case an increase of 840 per cent was noted in the case of a patient who had received 0.48 Gm. of theophylline ethylenediamine intravenously, followed in a few hours by 0.1 Gm. (1 c.c.) of mersalyl by vein. The "oral purine-intravenous mercury" regime as well as other combination regimes have been advocated in previous publications from this institution.<sup>8-9</sup>

On the wards of John Sealy Hospital and in the Cardiac Clinic, our custom in patients needing diuretics in addition to digitalis is to give a two- or three-day course of oral ammonium chloride (up to 6 or more Gm. per day) or theophylline ethylenediamine (up to 1 Gm. per day). This is followed by the intravenous administration of mersalyl-theophylline or mercurpurin. This is the classical method. In some cases of gastrointestinal intolerance, the use of a single dose of potassium or ammonium chloride or nitrate (2 Gm.) or theophylline ethylenediamine (0.4 Gm.) some two hours before the mercurial has been found to be as effective. This is the British method of Evans. In the most intractable cases of edema, theophylline ethylenediamine (0.48 Gm.), or a substitute, and the mercurial (2 c.c.) are given intravenously in the same syringe.

The results of diuretic therapy in the John Sealy Hospital were summarized,<sup>8</sup> and it was noted that decholin alone was relatively ineffective, but when injected intravenously (10 c.c. of 20 per cent) together with a mercurial a conspicuous diuresis resulted. Decholin also seemed to be particularly effective in diminishing liver engorgement.

Goodman and his co-workers<sup>10</sup> have reported a series of cases in which an accidental intravenous injection of  $7\frac{1}{2}$  grains of theophylline ethylenediamine one hour before intramuscular mersalyl (1 c.c.) started the reinvestigation of combined diuresis. They claim that the particular combination mentioned is the most effective. On the other hand, they claimed to have obtained greater diuresis from mersalyl alone than from mersalyl and aminophylline given simultaneously. Their explanation of the relative failure of the latter combination, as being due to quicker absorption and excretion of the mercurial, does not seem convincing. The same argument is used to prove the superiority of salyrgan-theophylline to salyrgan. Their study relied on daily weights only. We, on the other hand, by comparative studies in the same patient and in different patients, believe we have shown that some other combinations of intravenous diuretics are the most potent yet devised. At least, such combinations inaugurated brisk diuresis in cases which had proved refractory to all other methods.

#### METHOD

The patients used in this study were typically chronic cardiae in congestive heart failure unrelieved by bed rest and digitalis. They were continued on this regime, the usual low salt cardiac soft diet, an intake of approximately 1500 c.c. of fluids daily, and occasional oral acid salts or decholin. The diuretics were used in varying rotation. Mercurials in 2 c.c. dosage, theophylline ethylenediamine 0.48 Gm., and the two combined were so used intravenously.

In a few cases theophylline methyl-glucamine (glucophylline, Abbott), theophylline isobutanolamine (Merrell), theophylline isopropanolamine (National Drug Company), theophylline sodium acetate (Upjohn), and decholin (Riedel-de-Haen) were used in 10 c.c. dosage instead of aminophylline. In four cases aminophylline intramuscularly was injected two hours before the mercurial. Daily weights were unfortunately not obtained in all cases, for one reason, because of the absence of a scale capable of weighing bedridden patients. Because of that, comparative diuresis statistics are based on three-day percentage increases of urinary output, as in previous studies from this institution.<sup>5</sup> Ideally, of course, a weighed diet containing a known constant liquid and salt content, a bed scale capable of weighing to fractions of a pound, and more exact measurements of fluid output, including the amount lost in the stools, would be desirable.

It is worth mentioning that, while comparable statistics relating to each drug or combination are significant if enough cases are available, their comparison in individual patients is, perhaps, more significant. One drug or combination may fail in one patient and not in another because of individual differences in degrees of edema, digitalization, hypoproteinemia, cardiac decompensation, liver and kidney damage, and the like. For this reason the study of one patient, such as R. M. S. (see below), for several months, is quite rewarding.

#### RESULTS

Table I summarizes the diuretic effects obtained in 94 trials in 16 patients. It may be profitably compared with Table II (Drs. Herrmann, Dechard and Associates' 1940 series<sup>8</sup>). It will be seen, that, in general, mersalyl, fortified with xanthines or decholin, caused a greater diuresis than mercupurin or mersalyl-theophylline alone. The combination effects were at least additive, if not synergistic. Certain discrepancies are immediately discernible. The new smaller series show much lower values for diuresis in almost all subdivisions. It must be remembered, however, that for the sake of studying individual refractory edema cases, the cases chosen would show lower diuresis figures. In many of them mercurials alone caused hardly any diuresis; and that was even more true of the xanthines and decholin. The four cases receiving aminophylline intramuscularly prior to mercurials intravenously are too few to have significant statistical value. They are not strictly comparable to the cases in Goodman's series, since the order of administration in their cases was reversed.

The mercupurin combination responses are, in general, disappointing, except, possibly, for the theophylline sodium acetate combination. In the older series, too, mercupurin and aminophylline gave statistically lower diuretic responses than mercupurin alone. However, in the older experimental work<sup>8</sup> and in the new clinical series, the immediate diuresis (note first day figures) seemed to be increased by the combination. Moreover, in individual cases the combination responses were more striking than the figures would indicate. Against the validity of the statistics as a whole may be levelled the criticism that the probable error is high, due to extreme variations of response to the same drug or combination in the same patient and in different patients.

TABLE I  
SUMMARY OF DIURETIC EFFECTS

DRUG	NO CASES	% AVERAGE OUTPUT URINE INCREASE			
		1ST DAY	2ND DAY	3RD DAY	TOTAL 3 DAYS
"	27	242	57	46	393
"	15	247	18	23	278
"	8	41	109	61	261
ethylene diamine	8	147	10	1	358
acetate	5	628	38	69	735
"	2	530	0	20	350
"	3	310	75	0	385
Mercurpurin + decholin					
Aminoph. intramusc. then mercurpurin in-	2	211	377	40	628
traven.					
Mersalyl-theophylline + theophylline ethy-					
lenediamine	8	461	31	29	521
Mersalyl-theophylline + theophylline isabu-					
tanolamine	5	277	0	31	308
Mersalyl-theophylline + glucophylline	5	418	20	8	466
Mersalyl-theophylline + decholin	4	479	42	18	546
Aminophylline intramusc. then mersalyl in-					
traven.	2	425	18	5	448

TABLE II  
SUMMARY OF DIURETIC EFFECTS  
(Drs. Herrmann, Dechord and Associates' 1940 series)

DRUG	NO CASES	% AVERAGE OUTPUT URINE INCREASE			
		1ST DAY	2ND DAY	3RD DAY	TOTAL 3 DAYS
Mercurpurin	611	477	152	79	707
Salrgan	125	741	120	62	926
Salrgan and theophylline	75	335	107	42	484
Mercurpurin and aminophylline	10	415	154	33	562
Aminophylline	29	263	119	54	436
Theophylline sod. acetate	6	30	59	26	115
Decholin	22	83	34	11	128
Decholin and mercurpurin	7	770	101	7	878

As regards individual patients, combined diuretic therapy in all forms was more effective than mercurials alone in 11 out of 16 patients, questionably more effective in two more, and as effective in the other three. No toxic effects were noted except in one patient, who had reactions to either mercurpurin or aminophylline. The same patient tolerated mersalyl-theophylline well. Two cases may be singled out for demonstration.

CASE 1.—R. M. S., John Sealy Hospital No. 78659, was a 31-year-old rheumatic cardiac patient, with trivalvular stenosis, auricular fibrillation, cardiac cirrhosis, and ascites, who had been studied over four months. On digitalis, decholin, and acid salts by mouth he failed to respond to repeated mercurial and xanthine injections. After hospital admission, on intravenous salrgan-theophylline plus glucophylline he showed a marked diuresis, whereas the two separately had failed. The replacement of glucophylline by theophylline isobutanolamine resulted in a temporary increase in diuresis. Similarly, the substitution of decholin and later of intramuscular and intravenous aminophylline resulted in greater diuresis. Mercurpurin, likewise, while ineffective alone, caused marked diuresis when combined with theophylline ethylene diamine. In his case, at least, the rotation of diuretics and of their modes of administration appeared to be of therapeutic benefit (see Table III).

CASE 2.—R. G., John Sealy Hospital No. 59192, a 14-year-old Negro boy with constrictive pericarditis, probably of tuberculous etiology, presented marked ascites and gen-

eralized edema. Mercupurin and salyrgan-theophylline in 2 c.c. doses intravenously were at first effective, producing 24-hour urinary outputs up to 4000 to 5000 c.c. More recently, however, the diuretic responses were much lower. Aminophylline alone, in half gram doses intravenously, which had previously produced increased urinary outputs, roughly one-half those following mercurials, now failed completely. Mercupurin or salyrgan-theophylline at times caused no diuresis, and at other times, 24-hour urinary outputs up to 3000 c.c. The mercurials combined with aminophylline gave consistently higher results, with outputs in next 24 hours up to 4500 c.c. The same combined injections kept him in relative comfort in the Out-Patient Clinic, thereby avoiding frequent abdominal paracenteses.

TABLE III  
COMPARATIVE DIURESIS WITH MERCURIAL, XANTHINE, DECHOLIN AND COMBINATION  
DIURETICS IN CASE R. M. S.

DATE	DRUG I.V.	AMT.	% AVERAGE URINE OUTPUT INCREASE				
			1ST DAY	2ND DAY	3RD DAY	TOTAL 3 DAYS	WEIGHT LOSS
11/ 1/42	Mercupurin	2 c.c.	25	0	0	25	0
11/ 5/42	Salyrgan-theophylline	2 c.c.	30	40	30	100	-
11/12/42	Salyrgan-theophylline	2 c.c.	60	60	30	150	0
11/15/42	Glucophylline	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	300	0	0	300	8
11/20/42	Salyrgan-theophylline	2 c.c.	15	0	0	15	0
11/23/42	Glucophylline	10 c.c.	75	0	0	75	0
11/26/42	Glucophylline	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	300	100	0	400	-
12/ 1/42	Glucophylline	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	550	0	0	550	6
12/ 7/42	Glucophylline	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	600	0	0	600	4
12/10/42	Theophylline-isobutanolamine	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	650	0	0	650	5
12/16/42	Theophylline-isobutanolamine	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	250	0	0	250	0
12/21/42	Same as 12/16/42		220	0	0	220	0
12/27/42	Decholin	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	675	0	0	675	2
12/31/42	Same as 12/27/42		720	60	0	780	2
1/ 4/43	Decholin	10 c.c.	75	0	0	75	0
1/ 8/43	Decholin	10 c.c.					
	+ Salyrgan-theophylline	2 c.c.	475	40	0	515	2
1/15/43	Salyrgan-theophylline	2 c.c.	260	0	0	260	0
1/29/43	Aminophylline	0.48 Gm.					
	+ Salyrgan-theophylline	2 c.c.	510	0	0	510	2
2/23/43	Same as 1/29/43		675	0	0	675	3
3/ 3/43	Aminophylline i.m.	0.48 Gm.					
	+ Salyrg-theophyll. i.v. in 1 hour	2 c.c.	700	0	0	700	-
3/16/43	Same as 3/3/43		740	0	0	740	0

#### DISCUSSION

We may, we think, draw the preliminary conclusions that fortification of mersalyl, with theophylline ethylenediamine up to 0.5 Gm. of the latter results in greater diuresis than the addition of merely 0.1 Gm. of the xanthine. The latter is, of course, the amount in ordinary mercupurin or salyrgan-theophylline. These have already been proved superior to their predecessors, the mercurials without any theophylline added. In a study of creatinine clearances in 1937, Drs. Hermann and Decherd<sup>6</sup> mentioned that "in three instances in which mercupurin was fortified with aminophylline (0.24 Gm.) there was often a

very temporary decrease of filtration before diuresis began, but this was followed by a conspicuous rise in the glomerular filtration along with the characteristic mercurial impairment of tubular absorption. This fortified combination produced a more striking diuresis.

Studies are now being conducted, utilizing diodrast and inulin renal clearances, to measure renal blood flow and glomerular filtration, to study further to see if mercurials and xanthines have different renal loci of action. It has already been shown in previous blood volume studies of Drs. Dechard and Calvin and Herrmann<sup>11</sup> that while the mercurials cause mainly a decrease in blood volume by their renal effect, the xanthines may, after an initial drop, raise the blood volume conspicuously, in spite of any diuresis produced. This may be a possible extrarenal effect of the latter

### SUMMARY

The combinations advocated, then, are the logical outgrowth of past experience and should prove according to previous experimental evidence to be more effective as well as less toxic locally in and about the blood vessels and possibly on the renal tubular epithelium and the liver cell and on all tissues. It has been shown that xanthines protect somewhat against poisoning by organic mercurials.<sup>12</sup> The specific protection, if any, of the liver and kidney needs further proof.

The diminution of liver damage and congestion by decholin is still mainly a clinical surmise and should be further tested experimentally. It is suspected that the choleric action of decholin and increased circulation in the liver promote the excretion of mercury and fluid from the liver.<sup>11</sup>

Blood volume and renal clearance studies should be of help in the study of the mechanism of decholin diuresis. More clinical data are also needed.

Overdehydration, which may occasionally be accomplished by these powerful diuretic combinations, can certainly cause gastrointestinal disturbances, perhaps muscle cramps, and other so-called toxic manifestations seen after mercurials.

The most that can be claimed for any diuretic therapy is not prolongation of life, but relative well-being of the patient. The question of whether myocardial or renal damage is contributed to by repeated mercurial injections is not yet answered with finality. Theoretically, at least, the heart burden is lessened and digitalis action promoted by the diminution of refractory anasarcae.

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## HUMAN SERUM TREATMENT OF ATYPICAL PNEUMONIA

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THE fall and winter of 1942-1943 were characterized by the expected, or somewhat higher than expected, incidence of pneumonia among the personnel of the Glenview Naval Air Station. The disease occurred in both flying and nonflying personnel, with a higher incidence in the former. The usual typical pneumococcus lobar pneumonia was the exception rather than the rule. From Sept. 1, 1942, to May 1, 1943, there were 19 cases of lobar pneumonia and 61 cases of pneumonia of an atypical character, having all the aspects of a clear-cut symptom complex, which has already been described by a number of authors.<sup>1-5</sup> The U. S. Navy has recognized and identified this syndrome in its official nomenclature as "pneumonia, primary atypical, etiology unknown." Other diagnostic terms include "virus pneumonia," "atypical pneumonitis," "acute atypical bronchopneumonia," etc.

The clinical aspects of the syndrome are fairly characteristic; they are, in brief, an illness moderate in severity with gradual onset following a mild upper respiratory infection, severe malaise, nonproductive irritative cough, headache, chilliness, fever rarely exceeding 103° F., a relative bradycardia, normal to very slightly accelerated respiration, minimal or absent physical signs in the chest, a relative leucopenia, a few or no pneumococci in the sputum, characteristic roentgenographic findings diagnostic in themselves, and a negative response to sulfonamides or any other specific therapy for pneumonia.

Our first cases occurred in November and December of 1942. In approximately 65 per cent of the cases the pathology was in the lower lobes, and in over half of these in the right lower lobe. The fever usually fluctuated daily between 100° and 103° F. with a morning peak in more than one-half the cases. This was so characteristic that whenever a man with no physical signs had a fever peak in the forenoon and approached a normal temperature in the evening, we promptly x-rayed his lung fields, anticipating pulmonary infiltration of an atypical character. Chest pain was unusual in our cases. An occasional patient produced blood-tinged sputum. In no cases was there a true chill.

The febrile state lasted from five to eleven days, averaged about six days, and terminated by lysis. The roentgenographic findings persisted long after the fever subsided in all cases, and took upwards of four weeks to subside, occasionally longer. A surprising feature was the prolonged debilitation following a relatively mild disease state. Inasmuch as patients in military hospitals remain hospitalized until ready to return to active duty, there are more sick days for any given condition in military life than in civilian life. We

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found it necessary to keep our convalescent patients as long as fifty days before returning them to duty. This was, of course, particularly true of flight personnel.

The disease has been frequently reported to be of a highly contagious nature. No effort was made to segregate such patients in our wards, and no spread in the ward was observed.

Although fatal cases have been reported,<sup>1</sup> the prognosis in general is excellent. In our series there were no deaths directly due to pulmonary pathology, but one death occurred because of a complicating meningo-encephalitis (confirmed by autopsy). The only other complication we observed was an apparent relapse with a return of the nonproductive cough as the most severe symptom. However, we soon came to believe that this was really due to too early release to active duty.

Treatment with sulfonamides had no favorable effect on the course of the disease, and at times was actually a drawback. Many patients were made more uncomfortable because of nausea, and an already low leucocyte count was sometimes reduced to a dangerous level; accordingly these drugs were discontinued after the first few cases. Oxygen did not seem necessary and was used only once; in the fatal case wherein there was a marked cyanosis, the consolidation involving many lobes.

The only successful treatment so far described from the standpoint of shortening the number of sick days is the use of roentgen therapy. In 95.6 per cent of the author's cases so treated, the febrile period, total number of sick days, and the days for resolution, as proved by x-ray, were reduced roughly one-half.<sup>6</sup>

Wolf and Levinson<sup>7</sup> have reviewed the field of specific human convalescent and hyperimmune sera. They emphasized that "immunotransfusions should be reserved for diseases in which humoral immunity is a significant factor." However, they further state that "the persistence of circulating antibodies years after recovery from an attack of certain diseases is a noteworthy occurrence," and also point out that "normal adult serum provides an appreciable degree of protection against a variety of common diseases."

The natural thought occurred, therefore, that a specific humoral immunity might be set up, if only for a short time, in the symptom-complex described in this paper. Accordingly, patients were chosen from our first "wave" of cases as donors for a supply of serum. Two hundred and fifty cubic centimeters of blood were drawn from each man, no sooner than thirty days after the cessation of fever, and as soon thereafter as possible. This blood was then processed into serum, pooled, and bottled in 250 c.c. units.\* This was stored in a refrigerator to conserve antibody content. Only eleven such units were obtained. The small supply was due to the fact that many of our patients are rapidly lost to us through transfer to other places of duty.

*Results.*—Ten patients were treated with this first supply of serum. We selected an arbitrary initial dose of one unit (250 c.c.). In nine of the cases one unit was adequate to produce a satisfactory clinical response. In only one case was a second unit administered. The latter case will be described in detail,

\*The serum was processed by the Samuel Deutsch Serum Center, Michael Reese Hospital, Chicago, Ill. We wish to express our thanks to the director, Dr. S. O. Levinson, for the above, for his generosity in making available to us his wide experience with serum and plasma, and for his constructive interest and aid in this piece of work.



and any one of the other nine will serve for reporting, so typical was the response.

The immediate and late results both were most gratifying. In nine of the patients the fever fell to normal by crisis within twelve to eighteen hours and remained there. No unfavorable reactions were noted. Recovery from the acute symptoms followed rapidly with a moderate cough persisting for from ten to fourteen days. Subjectively, and compared to the untreated cases, the response was good; the patients felt well, wouldn't believe that they had pneumonia, and universally expressed a desire for early return to duty. This latter feeling stood out in marked contrast to the untreated patients who felt exhausted, and showed no inclination to return to duty for some weeks after their fever had subsided. The number of sick days in the untreated cases averaged approximately 39 days; that in the small number of treated patients, 22 days. This accounted for a saving of sick time of approximately 45 per cent.

The x-ray findings as a general rule were not so quickly affected. In several cases, treated within 24 hours of the onset, the consolidation seemed to resolve, as shown by x-ray, within the week. However, in the remainder of the cases, the findings on x-ray disappeared gradually over a period of two weeks. This was somewhat faster than in the untreated cases in which the x-rays were positive for from three to four weeks. However, early resolution was seen in an occasional untreated case, so these results are not considered conclusive.

CASE 1 (Control, Illustrative Case).—J. H. was admitted on 12/28/42 with complaints of chilliness, malaise, headache, sore throat, and cough. Admission temperature was 103.8° F., pulse 94, respiration 24. The physical examination revealed only an injected pharynx. X-ray disclosed an atypical consolidation in the left lower lobe. The leucocyte count was 6,200, with a normal differential. This patient received only symptomatic treatment. Fever subsided on the ninth day (Fig. 1), and the patient was returned to active duty with 38 days' total sick time.

CASE 2 (Serum-Treated, Illustrative Case).—J. M. was admitted on 2/15/43 with identical and characteristic complaints. Admission temperature was 102.2° F., pulse 88, respiration 26. The examination was essentially negative. X-ray revealed atypical consolidation in the lower right lobe. Two-hundred and fifty c.c. "virus pneumonia serum" were injected intravenously on the morning following admission.

The temperature fell by crisis to normal in less than 24 hours, the findings cleared on the twelfth day, and the patient was returned to duty with a total sick time of twenty days.

CASE 3 (Serum-Treated, Illustrative Case).—W. M. C. was admitted on 3/18/43, complaining of cough and nasal congestion for past few days. Admission temperature was 100.3° F., pulse 96, respiration 20. Physical examination revealed an injected pharynx and occasional fine crepitant râles in the left chest, posteriorly. Leucocyte count was 6,200. X-ray on the day of admission disclosed an atypical consolidation of the lower left lobe.

On 3/20/43 the temperature was 101.8° F. in the morning, and 250 c.c. of convalescent "virus pneumonia serum" were given intravenously. There was no reaction. The temperature fell to normal within 36 hours (Fig. 2) and remained there, except for one reading of 99.2° F. on the eighth day. This patient was discharged from the hospital on the sixteenth day, and returned to full duty on the twenty-third day. The x-ray findings were normal before discharge from the hospital.

CASE 4 (Serum-Treated, Two Doses Required).—E. C. was admitted on 3/16/43 with complaints of chilliness and feverishness for two days; coryza and cough for several days previous; admission temperature was 102.8° F., pulse 100, and respiration 20. Physical examination revealed only a mild injection of the pharynx and occasional crepitant râles

in the right chest posteriorly. Chest x-ray was normal. Leucocyte count was 7,650. X-ray on 3/19/43, a repeat roentgenogram, revealed an atypical consolidation of the right middle lobe. At this time the temperature was 103° F.

Two hundred and fifty cubic centimeters of convalescent "virus pneumonia serum" were given intravenously. Two hours later the patient experienced a chill lasting thirty minutes and the temperature gradually rose to 104.2° F. No such reaction had been observed previously, or has been seen since, and we believe it was due to faulty preparation of the tubing.

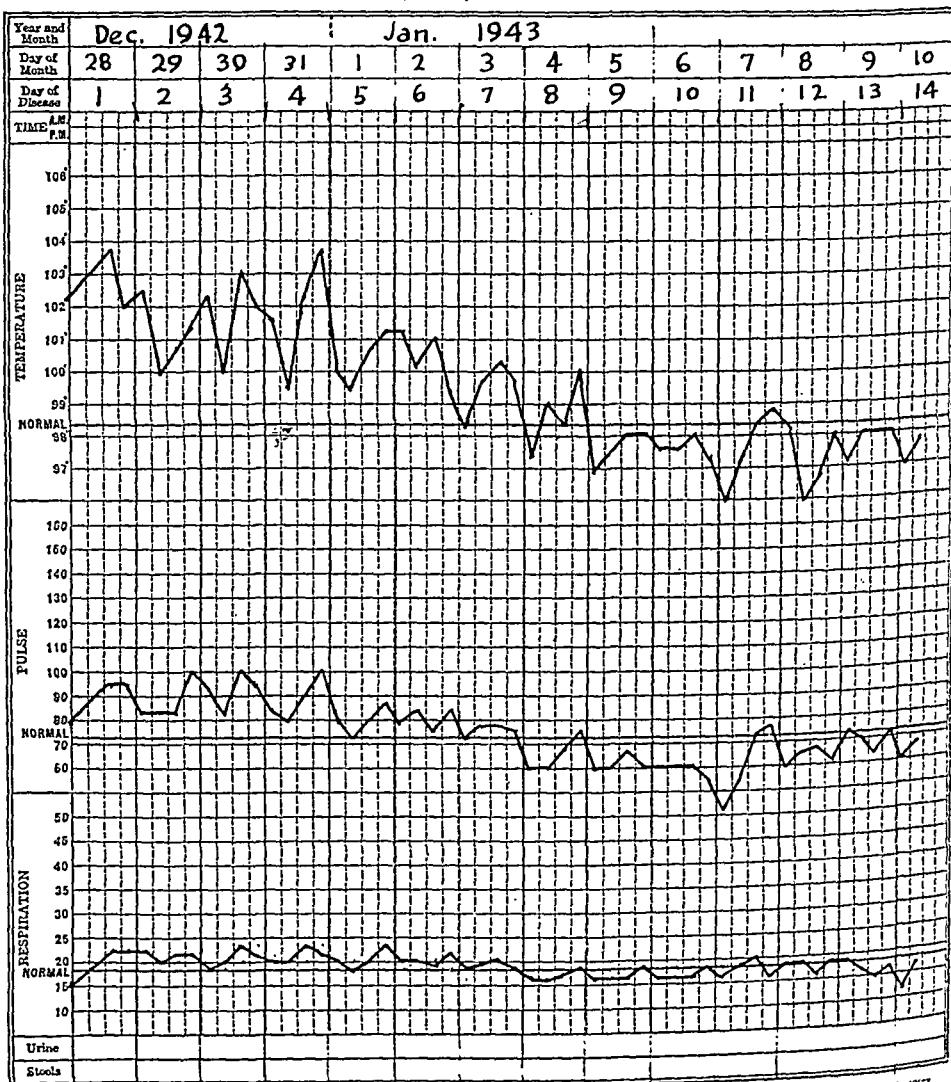
# NAVAL AIR STATION GLENVIEW, ILL. CLINICAL CHART

N. M. S.—Form Q  
(1939)

Page No. ....

Name J. D. H. Rate A. C.

Diagnosis Pneumonia, Primary Atypical Race Wh. Age 22



Day after Operation may be entered in red in the space for Day of Disease.  
Draw heavy lines to separate calendar days.

Fig. 1

The temperature fell slowly by lysis but approached 101° F. three times in 72 hours. Therefore, on 3/22/43, a second unit of serum was given. There was a prompt return to normal temperature (Fig. 3), and this patient was sent to active duty on 4/8/43, a total of only 23 sick days.

*Discussion and Summary.*—Although this disease has a low mortality, the long hospital stay makes this no ordinary military liability. The disease inci-

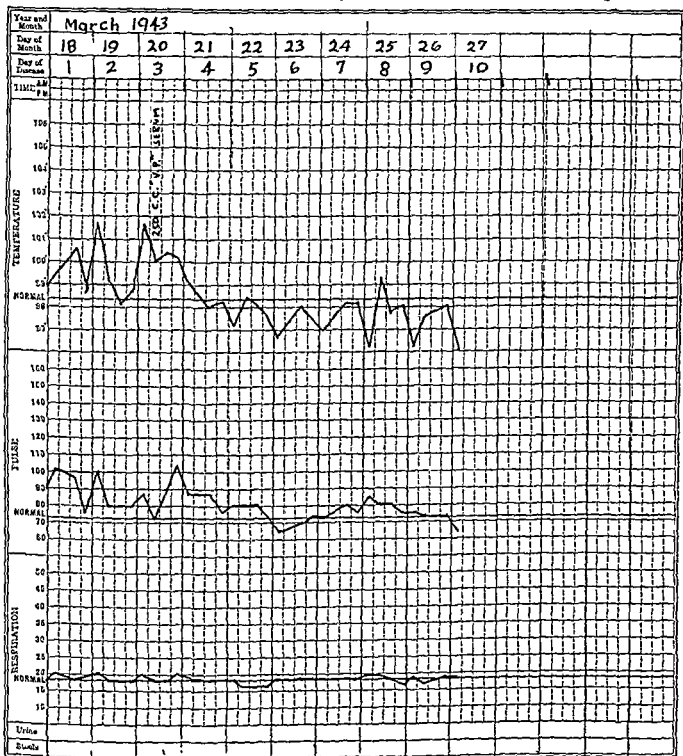
NAVAL AIR STATION  
GLENVIEW, ILL.  
CLINICAL CHART

N. M. H.—Form Q  
(10-2)

Page No. \_\_\_\_\_

Name W. M. C. Race A. C. M. M.

Diagnosis Pneumonia, Primary Atypical Race Wh Age 24



Day after Operation may be entered in red in the space for Day of Disease.  
Draw heavy lines to separate calendar days.

10-10128

Fig. 2

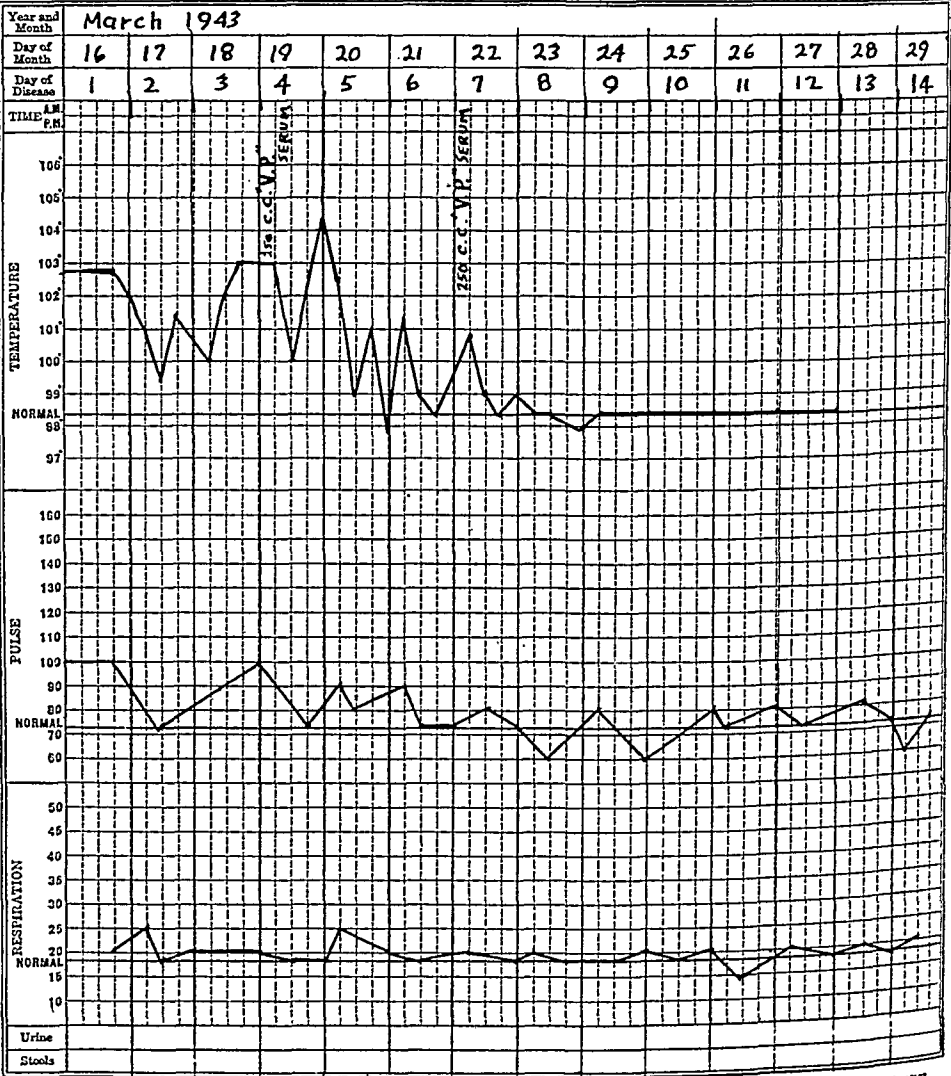
NAVAL AIR STATION  
GLENVIEW, ILL.  
CLINICAL CHART

N. M. S.—Form Q  
(1939)

Page No. ....

Name E. C. Rate 5'6"

Diagnosis Pneumonia, Primary Atypical Race Wh. Age 29



Day after Operation may be entered in red in the space for Day of Disease.  
Draw heavy lines to separate calendar days.

Fig. 3

dence and severity have increased in the past several years. There is evidence to support the belief that the coming winter, particularly with large concentrations of people and mass shifting of population, may bring an epidemic of atypical pneumonia, larger in scope and more severe in nature than has been seen previously. In the absence of specific therapy, the treatment with convalescent serum yielded interesting results. Cases of atypical pneumonia which did not receive serum displayed fever lasting from five to eleven days, which dropped to normal by lysis. Convalescence was prolonged; lasting on an average of 39 days. Nine out of ten patients treated with convalescent "virus pneumonia serum" had a temperature drop to normal by crisis, and a reduction in the convalescent period to an average of twenty-two days. These patients responded to one dose of 250 c.c. of serum. Only one patient did not respond in this way, but showed a beneficial effect following a second dose of serum. Despite the rapid temperature drop to normal, and a very marked decrease of convalescent time, the days for resolution as shown by x-ray, were not shortened in proportion, although some reduction was noted.

The cases treated occurred in late winter and spring and were from our second "wave" of cases. Cases for treatment were neither chosen in succession nor in alternation with untreated cases. Rather we treated only those cases who had the most clear-cut syndrome and who seemed to be the most severely ill from a clinical standpoint. The last case treated with convalescent serum occurred in late March. We continued to see patients with this syndrome all through the month of April, which was unusually cold and wet this year in this locality.

In these late cases, due to the lack of convalescent "virus pneumonia serum," pooled adult serum in the same dosage was employed. The response in these few patients seems to be comparable to that obtained with the convalescent serum. This may be due to the fact that a large percentage of the normal adult population was antiviral substances against this, as yet unisolated, causative agent. This is the experience with certain other virus diseases. Further study along this line seems indicated, particularly in view of the fact that pooled adult serum or plasma can be obtained with relative ease.

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# A CASE OF MENINGITIS IN A PREMATURE INFANT DUE TO A PROTEOLYTIC GRAM-NEGATIVE BACILLUS\*

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THE occurrence of meningitis is less common in the newborn infant than in older children, and the incidence of the disease in premature infants is rare. Koplik<sup>1</sup> observed meningitis in twelve infants less than three months of age, and only one case in a prematurely born infant was reported. This was an infant of seven months' gestation that died of *Streptococcus meningitis* at the age of six weeks. Fothergill and Sweet<sup>2</sup> in a review of 705 cases of meningitis in children up to twelve years of age presented only three cases in premature infants. One was caused by *Pneumococcus* in an infant of eight months' gestation; one occurred in an infant delivered by cesarean section in the eighth month of gestation and was caused by an organism of the *Alcaligenes* group; the third case reported by them occurred in a premature infant of seven and one-half months' gestation and was due to *B. acidi lactici*. All these infants died. Craig<sup>3</sup> reported 21 cases of neonatal meningitis. Seven of these occurred in infants born four weeks prematurely, and 8 in infants born six or more weeks prematurely. None of these survived. Kutscher<sup>4</sup> reported a case of *Alcaligenes bookeri* meningitis, with recovery, in a four-month-old infant that had been born two months prematurely. No chemotherapy was used. In a review of 42 cases of meningitis in the newborn infant, Barron<sup>5</sup> did not report any cases in prematurely born infants. Holt<sup>6</sup> reviewed a series of 300 of his own cases of meningitis in infants up to three years of age. Only 11 were in infants under three months of age, none of which were premature. Borovsky<sup>7</sup> reported in 1930 that out of 190 patients with *Meningococcus meningitis* admitted to the Cook County Hospital in Chicago within a period of eighteen months the youngest was three months old. Cooke and Bell<sup>8</sup> in a report on the incidence of meningitis in early infancy reported only 10 cases under three months. None were in prematures. In a series of 136 cases of meningitis reported by McLean and Caffey<sup>9</sup> the youngest case included was a twenty-three-day-old full term infant. In 1926 Neal<sup>10</sup> reviewing the literature on meningitis caused by the bacilli of the colon group presented 118 cases and none were in prematurely born infants. In 1935 Ravid<sup>11</sup> reviewed 121 cases of meningitis seen in the New York Post-Graduate Hospital in the four-year period from 1928 to 1931, and only eight were in infants under three months. None occurred in prematures. Cooperstock<sup>12</sup> reported 14 cases of meningitis under one year of age and none were in premature infants.

We are reporting a case of meningitis due to a proteolytic Gram-negative bacillus in a prematurely born infant treated successfully with sulfathiazole. This case is of particular interest because of the unusual organism involved. the use of chemotherapy, and the subsequent recovery.

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## CASE REPORT

J. P., a nine-day-old premature female infant, was admitted to the Extramural Premature Nursery of the Long Island College of Medicine Division at Kings County Hospital on Dec. 19, 1942, because of jaundice, discharging eyes, and weight loss.

The family history was noncontributory. The mother was 24 years old, and there had been no previous pregnancies. The antenatal history was interesting. When the gestation had reached four months, the mother who was unaware of the pregnancy underwent an operation for a uterine tumor. The pregnancy was discovered and allowed to continue. The pregnancy progressed uneventfully until the eighth month when the mother went into spontaneous labor and delivered a 5 pound, 5 ounce, female infant by vertex presentation at another hospital. At the time of delivery the mother was in excellent health.

After birth the infant fed poorly despite changes in the formula. During the week prior to admission the weight fell to 4 pounds and 13 ounces. On the fifth day the child became jaundiced and appeared weak. On the day before admission the infant developed a purulent nasal discharge and edema of the periorbital region. A diagnosis of acute ethmoiditis and sepsis was made and the child transferred to Kings County Hospital.

On admission the child was nine days old. She appeared jaundiced, weak, and had a feeble cry. The admission temperature was 95° F. and the weight was 4 pounds and 12 ounces. There was a moderate amount of nasal discharge, but no periorbital swelling was noted. There was a slight discharge from both eyes. The buttocks were red and inflamed. The umbilical stump was moist.

The infant was admitted to the Extramural Premature Nursery and placed in an incubator. A urine analysis on the second hospital day was normal. On the same day a blood count showed 13 Gm. of hemoglobin and 14,000 white blood cells per cubic millimeter with 46 per cent polymorphonuclear cells and 54 per cent lymphocytic cells. The icterus index was 66 units. Cultures of the discharge from the eyes revealed diphtheroids. Culture of the drainage from the umbilical stump was positive for *B. proteus vulgaris*, *Staphylococcus albus*, and a few colonies of nonhemolytic *Streptococcus*.

For the first week the patient did poorly. All feedings were taken with difficulty and the weight continued to fall despite gavage. On the ninth day in the hospital it was observed for the first time that the child held the head retracted and had a high pitched cry. At the same time the temperature rose to 102° F. and the infant appeared much weaker. A lumbar puncture was performed with difficulty and blood-tinged fluid was withdrawn. The presence of red blood cells in the cerebrospinal fluid was attributed to trauma in performing the tap. A specimen of the fluid was sent to the laboratory for culture and reported positive for Gram-negative bacilli.

On the eleventh day the spinal tap was repeated and cloudy xanthochromic fluid was obtained. The fluid contained 500 white cells per cubic millimeter with 95 per cent polymorphonuclear leucocytes. The sugar content was too low to estimate quantitatively. A culture of the fluid was again reported positive. A blood culture taken on the same day was also positive for the same organism. On the twelfth day the child developed twitchings of the arms which lasted only a short time and did not recur. On the sixteenth hospital day the spinal fluid contained only 30 cells per cubic millimeter with 50 per cent lymphocytes. The sugar was 17 milligrams per 100 c.c. A culture of the fluid was still positive. On the twenty-second day the fluid was clear and the cell count was 215 per cubic millimeter with 100 per cent lymphocytes. The sugar had risen to 25 milligrams per 100 c.c. A culture was sterile for the first time. A blood culture was likewise sterile. A final spinal tap on the thirty-first day was again sterile. The cell count was 65 per cubic millimeter. The sugar was 58 milligrams per 100 c.c.

Several attempts to isolate the same organism from the stools were unsuccessful.

On the tenth day the patient was started on sulfathiazole by mouth. She received 0.25 Gm., daily until the thirteenth day when the dose was increased to 0.50 Gm. daily. This was continued until the thirty-second hospital day. During this course hemotherapy the blood titers ranged from 1.3 to 6.3 milligrams per 100 c.c.

On the third, thirteenth, and thirty-first days the infant received transfusions of whole citrated blood.

For the first three weeks the child was weak and pale. The temperature ranged from 99° F. to 102° F. At the end of this time it returned to normal and with the exception of an isolated rise on the thirty-first day following a transfusion it remained normal. The admission weight of 4 pounds and 12 ounces fell to 4 pounds and eight ounces. On the sixth day breast milk feedings were started and on the ninth day the weight began to rise. The gain was progressive. The icterus gradually subsided.

On the fortieth hospital day, at the age of forty-nine days, the infant was discharged in excellent condition weighing six pounds and four ounces.

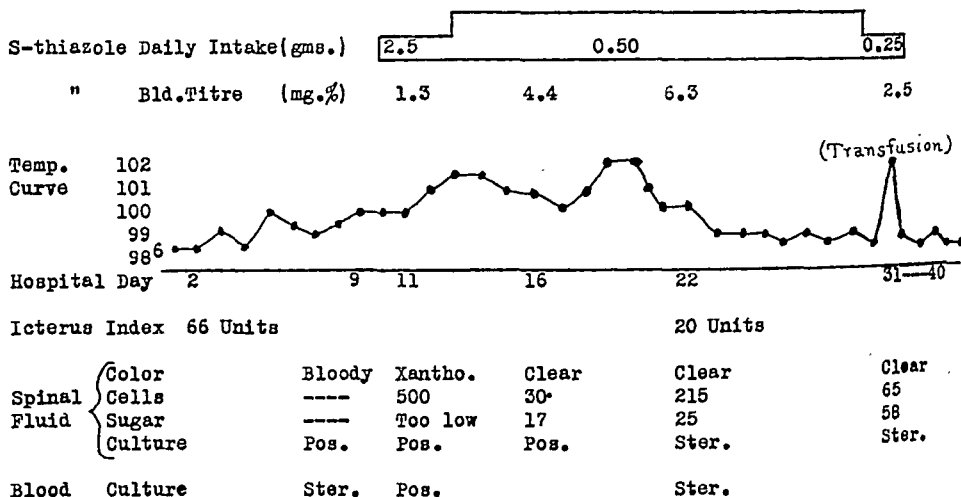


Chart I.

#### BACTERIOLOGY

The microorganism recovered upon one occasion from the blood and from three consecutive cerebrospinal fluids of the patient, J. P., was a nonmotile, short, straight Gram-negative bacillus with rounded ends. The bacilli occurred singly and were uniform in appearance in young cultures, but long filamentous forms were found with regularity in old fluid cultures. No branching or involution forms were observed. The organism was not acid-fast and stained readily and evenly with all the usual dyes. Flagella, endospores, capsules, and metachromatic granules were absent.

Growth was abundant on all ordinary media and was not enhanced by the addition of accessory substances. Surface colonies on agar were discrete, circular, low-convex, and amorphous with smooth surface and entire edge. There was no hemolysis or discoloration on rabbit blood agar. After 24 hours' incubation at 35° C. the diameter was 2.5 to 3.0 mm. and the color was gray with a yellowish tint. On gelatin, colonies were larger in size, irregular in shape, and medium granular in structure. Liquefaction was rapid and in stab culture was infundibuliform, extending to the base of the inoculation. Löffler's blood serum was partly digested in five days.

Chromogenesis on extract agar and on glycerol peptone agar was limited to the slight yellow of young cultures which deepened to lemon-yellow after long incubation. Neither fluorescence nor diffusing pigment was seen at any



time. Growth on potato was abundant and the yellowish-gray color present early became brown with browning of the medium in two weeks. These yellow and brown pigments were not soluble in water, alcohol, chloroform, or weak alkali.

The organism was aerobic, facultative anaerobic with an optimal growth temperature of 35° C. though it grew easily at 22° C. It was killed by exposure in a water bath to a temperature of 56° C. for 30 minutes. Growth was not inhibited by crystal violet, brilliant green, or sodium tellurite, but was prevented by bile salts in the concentrations commonly used in differential media.

Litmus milk showed an alkaline reaction; litmus was reduced except for a blue surface ring; a soft coagulum with serum layer was formed and subsequently peptonized. Indole, acetyl-methylcarbinol, and hydrogen sulfide were not produced. Nitrates were not reduced. Methyl-red and citrate agar tests were negative. Starch was hydrolyzed and tests for catalase and methylene-blue reductase were strongly positive.

No visible acid or gas was produced over a period of four weeks in peptone-carbohydrate broths; however, when the substrates were incorporated in a synthetic medium (0.2 Gm. magnesium sulfate, 0.1 Gm. calcium chloride, 0.2 Gm. sodium chloride, and 0.2 Gm. dipotassium phosphate per liter of distilled water)<sup>17</sup> with brom cresol purple indicator definite reactions as shown by distinct color changes were obtained. Glucose, mannitol, lactose, maltose, glycerol, salicin, levulose, trehalose, mannose, and dextrin were fermented with the formation of acid only in seven to ten days, while xylose, sucrose, arabinose, raffinose, cellobiose, rhamnose, adonitol, galactose, inositol, inulin, dulcitol, and sorbitol were not affected. It is interesting to note that while the methyl-red test was negative, the final pH of glucose-synthetic medium was 4.2.

Macroscopic agglutinations were negative with all available diagnostic immune serums in dilutions 1:20 through 1:320. Antiseruma of the *Shigella*, *Eberthella*, *Salmonella*, and *Brucella* groups, and *Pasteurella tularensis* were employed.

In a small series of laboratory animals, pathogenicity was found to be of a low degree. Guinea pigs survived an intra-abdominal inoculation of 1 c.c. of an 18-hour broth culture. Of 6 mice inoculated intra-abdominally with 0.5 c.c. of the culture, one appeared sick on the eleventh day and succumbed on the twelfth. The test organism was recovered from the heart's blood, pericardial exudate, spleen, and from small abscesses in the liver and mesentery. A second mouse became sick on the twenty-seventh day and was sacrificed. Cultures of the heart's blood were negative, but the enlarged spleen and an abscess in the liver yielded pure cultures of the bacillus. The remaining 4 mice survived. The organism was not altered in any way by mouse passage.

Among the organisms causing meningitis many varieties of Gram-negative bacilli have been reported. Of these, with the exception of *Hemophilus influenzae*, bacilli of the colon group were noted most frequently especially in infants. Barron<sup>8</sup> found that in 42 cases in infants under 5 months, 14 were caused by *Escherichia coli* and one each by Friedländer's bacillus, typhosus, *laetis aerogenes*, *pyocyaneus*, and *H. influenzae*. Neal<sup>10</sup> reported 50 cases under

3 months of which 5 were due to Gram-negative bacilli, 3 to *E. coli*, and 2 to *H. influenzae*. The remainder were tuberculous or coccal. Of 21 neonatal meningitides reported by Craig,<sup>3</sup> 10 were due to *E. coli* and 11 were coccal. Rauch and Krinsky<sup>13</sup> in 1940 concluded that although coli meningitis is rare when all meningitides in infancy and childhood are included, "coliform bacilli" are the commonest cause in infants under three months. Case reports have cited as other etiologic agents of meningeal infection, bacilli of the *Salmonella*, *Proteus*, *Brucella*, *Pasteurella*, and *Alcaligenes* genera and Koch-Weeks bacillus of the *Hemophilus* group. In others the causative bacillus was not identified.

The difficulties to be encountered in identifying a culture without accepted suitable criteria are obvious. Antigenic analysis is not always possible, and while an organism may seem to belong in a given classification with morphology as a criterion, it may be excluded from that same classification on the basis of physiologic characteristics. In the case herewith reported, the authors believe that any classification of the organism which for convenience is referred to as the "J.P." bacillus would be arbitrary and possibly erroneous, and they prefer to present the bacteriology in detail with comment upon similar described species. As tests for carbohydrate fermentation by these organisms were carried out in peptone-containing preparations used in routine investigations by most workers, it is necessary to use the negative reactions obtained with the "J.P." bacillus in this type of medium as a basis for comparison and to regard it as a "nonfermenter."

In 1933 Fothergill and Sweet<sup>2</sup> included in 9 cases of "colon group" meningitis one in which the bacillus recovered fermented no sugars and produced a slight yellowish pigment. Other data were not given.

Hazen and Mortillaro<sup>14</sup> in 1936 reported a fatal septicemia in an adult female caused by a "Hitherto Undescribed Microorganism of the *Alcaligenes* group." Their bacillus, although nonmotile, M.R.-negative, V.P.-negative, indole-negative and nonfermenting, was not proteolytic, did not change the reaction of litmus milk, and had variable pathogenicity for guinea pigs but none for mice.

A motile proteolytic member of the genus *Alcaligenes*, *A. bookeri*, an intestinal inhabitant, was recovered from a case of meningitis by Kutscher<sup>1</sup> in 1937. *A. bookeri* differs from the "J.P." bacillus in being motile and reducing nitrates to nitrites. There is a tendency in many laboratories to classify readily cultivable nonpigmented and nonfermenting Gram-negative bacilli, especially those of fecal origin, as *Alcaligenes*. Although this seems a possible genus in which to place the "J.P." bacillus, we do not feel justified in doing so. The genotype, *A. fecalis*, has always been defined as peritrichous and rod-producing, neither acid nor gas in any of the usual test substances. The possibility that *A. fecalis* might utilize glucose in such a manner that the reaction could not be detected by the usual culture methods was investigated by Conn.<sup>15</sup> From tests on synthetic media of nonfermenting soil bacilli and 23 strains of nonfermenting intestinal bacilli from 6 different sources, he concluded that, "The weight of evidence from this comparison of cultures is that *A. fecalis*, as generally recognized, does not grow on the particular synthetic medium

selected, and unlike the soil forms, cannot make use of glucose on such a medium. There is no evidence at hand to indicate that *A. fecalis*, as represented by the bulk of cultures received, can grow without organic nitrogen."

The "J.P." bacillus is analogous to the soil forms and inconsistent with the intestinal forms in its ability to utilize glucose and inorganic nitrogen as indicated by abundant growth on this medium.

One of the nonintestinal *Alcaligenes*, *A. marshalli*, described in Bergey's Manual<sup>10</sup> as a proteolytic nonmotile milk bacterium with yellow chromogenesis, parallels closely the "J.P." bacillus in the morphologic and physiologic properties recorded, but the description is meager and the species is not well defined. Conn<sup>15</sup> in a treatment of the "Validity of Genus *Alcaligenes*" has recommended that this species, along with other nonintestinal *Alcaligenes*, be transferred to "an appendix of indefinitely described species."

Of bacilli reported in cases of meningitis and described as forming no visible organic acid from carbohydrates, the "J.P." bacillus resembles most closely the one reported by Cooke and Bell<sup>8</sup> in 1922 as a "distinctly new type of organism" and for which they proposed the name "*Bacterium elasticum*." With the exception of *B. elasticum*'s viscid type of growth and its greater virulence for mice, the two bacilli possess like properties. By intra-abdominal injection *B. elasticum* was fatal to mice in twelve hours, and a small capsule was demonstrated in smears of the mucoid peritoneal exudate. The organism was nonmotile, strongly proteolytic, peptonized milk with little change in reaction and had no virulence for guinea pigs. There was no fermentation with gas of any sugars, but small amounts of acid with some were detected by changes in pH from 7.5 to 7.0-6.6, but never lower. These observations were made from peptone-containing cultures. In this type of medium we also noted that with some sugars pH reactions were lowered slightly.

Elrod and Braun<sup>17</sup> with synthetic preparations found that *Pseudomonas aeruginosa* attacked glucose, xylose, and arabinose, although Topley and Wilson,<sup>18</sup> Sandiford,<sup>19</sup> and Zinsser and Bayne-Jones<sup>20</sup> stated that glucose only was fermented. These studies suggest the possibility that some of the so-called "inert" proteolytic alkalizing bacteria might prove to be acid-producing if methods other than those in general use were applied.

Similarities of a physiologic nature may indicate a relationship between the "J.P." bacillus and the *Pseudomonadeae* consisting of the pyocyanic and fluorescogenic bacilli and the plant pathogens, *Phytomonas*. Many of the *phytomonas* form green fluorescent pigment, but others may be yellow or white. It is significant to us that Elrod and Braun<sup>17</sup> found 2 strains of *Pseudomonas polycolor* to be indistinguishable from strains of *Ps. aeruginosa* and that many authorities agree that differentiation of *Ps. pyocyanea* and *Ps. fluorescens* is often impossible. The pathogenic potentialities of *Ps. aeruginosa* are well known, and it has been reported frequently in cases of meningitis. Although Bergey et al.<sup>16</sup> state that this organism produces indole, all of the fifty strains tested by Sandiford<sup>19</sup> were negative; indole was not formed by any of the pathogenic strains studied by Zinsser and Bayne-Jones<sup>20</sup> nor by the 15 strains of Elrod and Braun.<sup>17</sup> The latter authors state that rapid liquefaction of gelatin and failure to form indole are considered characteristic reactions of the species,

and authorities do agree in describing *Ps. aeruginosa* as motile and capable of liquefying gelatin.

Dissociants may arise, however, which show loss or alteration of one or more essential characteristics. Sommensehein (Zinsser and Bayne-Jones<sup>20</sup>) described a mucoid encapsulated variety in 1927. Blane (quoted by Hadley<sup>21</sup>) produced by the "modifying" action of filtrates a strain in which both pyocyanine and fluorescein were suppressed with a concurrent loss of the power to liquefy gelatin. Gessard (Hadley<sup>21</sup>), by the use of heat or animal passage, obtained from a strain with both pigments one producing fluorescein only. Hadley<sup>21</sup> described variform types derived from a single lysogenic strain. His resistant nonlytic "R" type, capable of reproducing its own type only, was always nonpyocyanigenic and sometimes achromogenic. Loss of pigment was accompanied by depression of proteolytic activity. This correlation was not found in the "J.P." bacillus in which feeble pigmentation was associated with a strongly active proteolysis.

It is well recognized that under artificial conditions bacterial transmutation may give rise to types which scarcely resemble the "parent" strain. In medical bacteriology the primary isolation of atypical strains is not uncommon and presents only temporary taxonomic difficulty if the dissociation is of the reversible type. If, on the other hand, the variant were of the relatively permanent type retaining its aberrant properties under diverse conditions, classification of the variant and identification of the "original" species might prove impossible. Over a period of five months there has been no change in the characteristic morphology and reactions of the "J.P." bacillus. Although it is our impression that a close relationship to those allied families, the Rhizobiaceae and the Pseudomonadaceae, is probable, a correct identification cannot be made at this time.

#### SUMMARY

A case of meningitis due to a proteolytic Gram-negative bacillus in a seven-month premature infant is reported.

The successful use of sulfathiazole is described.

A detailed bacteriologic investigation of the organism with a discussion of similar Gram-negative bacilli is presented.

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## STERNAL BONE MARROW IN THE AGED\*

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**D**URING the past few years a great deal of attention has been focused upon the medical problem of the aged. This is due in no small part to the fact that improved methods of preventive and therapeutic medicine have increased life expectancy by sixteen years.

The most pronounced pathologic changes seen in the aged are those of atrophy. The internal organs decrease in size, the liver becomes a flabby shrunken organ, and the bones become rarified.

The formative tissue of the blood, the bone marrow, is the most active tissue of the body, from infancy to old age. The red and white cells are being continually destroyed and replaced, and the bone marrow must always be ready to regenerate at great speed, to meet any unforeseen demand for granular or erythroid cells.

It would, therefore, seem of importance to study the bone marrow in the aged under varying conditions, especially in regard to possible atrophic change.

To accomplish this, one hundred old patients, both male and female, sixty-five to ninety years old, were selected for study from the clinic wards of a large city hospital.

Sternal punctures were done in the usual way. A small spot of iodine was painted on the midsternal area opposite the second interspace. Novocaine was injected, and a 16 gauge needle was tapped into the marrow cavity. Four c.c. of marrow were aspirated and placed into a tube prepared previously by evaporating one c.c. of 1.4 per cent sodium oxalate to dryness.

Total nucleated cell counts were done. The material was then centrifuged, the plasma was discarded, and cellular material recentrifuged in a thin tube. The marrow layer was then removed and smears were made and stained thirty minutes by Wright's stain.

Differential counts of the smear were not done owing to the inaccuracy of the method. Instead, the smear was examined just as a section of tissue is examined and judged in regard to megakaryocytes, granular cell, and erythroid activity.

At the same time, hemoglobin and white cell determinations were done on the same patients to check on the concomitant changes in peripheral blood.

A study of the experimental data reveals that the bone marrow remains relatively normal in the aged. This is rather surprising at first since the general tendency in old age is one of atrophy. On second thought, however, it is

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easy to see that life could not continue very long after the bone marrow became atrophic, and therefore survival to old age requires a good functioning bone marrow. In some of the very old patients the cellular activity of the marrow was not only normal, but definitely increased.

It is also of interest to note that most of the old patients had an increased white count. In some cases this was due to some concomitant pathologic process, but the response was usually one of increase rather than decrease of white cells.

This observation coincides with our findings in animals. In this work we found that rats which lived longest had normal higher white counts than those with shorter life span.

It is possible that the same pertains to human beings and that one of the reasons that some persons live longer than others is that they are endowed with a better bone marrow.

# CLINICAL CHEMISTRY

## ALLOXAN DIABETES IN DOGS\*

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**S**HORTLY after Bailey and Bailey<sup>1</sup> reported their production of diabetes in rabbits by alloxan, I undertook a number of experiments intended to elucidate whether the same was also possible in dogs. When the results that are now being published had already been found, Dr. C. C. Bailey, in a private letter, kindly made me aware of the fact that alloxan diabetes in dogs had already been reported by Brunschwig et al.<sup>2</sup> This knowledge gave me the impression that since alloxan diabetes in dogs is already a published fact, my results were devoid of any special interest and did not deserve publication until they were followed by many others, as a result of further experimental work on the same subject. However, a recent reconsideration of the facts that I had found a few months ago makes me feel now that, aside from the scientifically irrelevant detail of priority in the publication of the mere fact of alloxan diabetes in the dog, there is nothing, either in Brunschwig, Allen, Goldner, and Gomori's first report,<sup>2</sup> or in the more recent paper by Brunschwig, Allen, Owens and Thornton,<sup>3</sup> to deprive my results of whatever interest they might have in themselves, and since I think that, in fact, they may be found of some interest, I made up my mind to submit them for publication.

### METHODS

Alloxan was injected, as will be specified, into five dogs. Except in one of them that died in less than half an hour, blood sugar was determined before the injection, and two or more times afterwards, within the next twenty-four hours. The animals were continuously watched for five to nine hours after the injection. Three of the dogs lived for several days, and one of them is still alive, five months after the injection. Unless otherwise stated, the dogs were fed, once a day, an unselected, mixed, and copious diet (restaurant waste); several fasting blood sugar determinations were made on different days; urine was collected, sometimes in 24-hour periods, and, at other times, two portions were separated in the 24 hours, one a few hours after feeding and another one that might be taken as fasting urine. Several urine examinations were made for dextrose and ketonic bodies. From two of the dogs, pancreatic biopsies were taken, when alloxan diabetes had been produced in them. From the other three, pancreatic specimens were taken post mortem. The histologic studies that have been performed by Dr. V. Suárez-Soto of this university will be published elsewhere and

\*From the Laboratory of Physiology, University of Puebla.

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Alloxan was kindly supplied by Dr. F. Lehman of the "Laboratorios Hermona S. A." of México City.



will be given only the slightest consideration here. Dextrose tolerance tests were performed on two of the diabetic animals; for comparison, a similar test was performed on one of the dogs of this series before the alloxan injection was administered, and also a dextrose tolerance test was performed on a sixth intact dog, as a normal control.

Blood sugar determinations were made by a slight personal modification<sup>4</sup> of the well-known Hagedorn-Jensen method. Urinary sugar was tested with the Benedict qualitative reactive; and ketone bodies by the Legal reaction; results for both, when positive, are given by means of plus signs from one to four. To avoid repetition, it will be stated only here in the article, that blood sugar figures are given in milligrams of dextrose per 100 c.c. of whole venous blood. In Tables III, IV, and VI, "fasting blood sugar" means, not only fasting, but before any injection is given. In Tables I and II, "before" means just before (within three minutes) the beginning of the injection of alloxan or the beginning of the milk with dextrose drinking by the animal, respectively. In Tables I and VII, the time figures, except when otherwise stated, mean moments at the end of the stated time lapses, after the injection of alloxan, counted from the end of the last injection. In Table II, the time figures mean moments of blood sampling, at the end of the stated time lapses, counted from the moment the animal finished drinking the milk with dextrose.

#### RESULTS

For the sake of saving space, together with clarity, the results are given only in tabulated form.

TABLE I

EARLY EFFECTS OF ALLOXAN UPON BLOOD SUGAR. THE INJECTIONS TO DOG 5 WERE GIVEN DURING THE FOLLOWING INTERVALS: FOUR DAYS BETWEEN FIRST AND SECOND SUBCUTANEOUS; THREE DAYS BETWEEN THE SECOND SUBCUTANEOUS AND INTRAVENOUS. WHEN GIVEN THE ALLOXAN INJECTIONS, ALL DOGS, EXCEPT DOG 3 (SEE TABLE V), HAD BEEN FASTED FOR NOT LESS THAN 20 HOURS AND WERE KEPT FASTING UNTIL AFTER THE LAST BLOOD SAMPLE, ON THE SAME DAY OF THE INJECTION, WAS TAKEN, WHEN THEY WERE FED

DOG NUMBER:	1	2	3	5	5	5
Milligrams of alloxan injected per kilogram:	100	100	300	75	75	200
Kind of injection:	intra-venous	intra-venous	intra-venous	subcu-taneous	subcu-taneous	intra-venous
Before:	102	105	93	110	105	121
15 minutes	102					
30 minutes			90			
2 hours	140			91		
2½ hours		106				99
3½ hours	124					
4 hours			220	83		
5½ hours					105	
8 hours				81		
8½ hours	75					76
9 hours		87				
24 hours	343	125				281

#### COMMENT

*The Production of Diabetes in Dogs by Alloxan Has Been Confirmed.*—Not only glycosuria and hyperglycemia in Dogs 1, 2, and 5, but also polydipsia and polyuria in Dogs 1 and 5 and severe weight loss in Dog 1 can leave no doubt

about this point. Further evidence is afforded by the dextrose tolerance tests (Table II), that of Dog 1 corresponding to a rather severe diabetes and the first test of Dog 2 corresponding to a very mild one.

*Curability of Alloxan Diabetes.*—Dog 2, after exhibiting mild glycosuria and hyperglycemia for a few days, as a result of an injection of alloxan, and giving a mild diabetic dextrose tolerance test, not only had, shortly afterwards, a permanently sugar-free urine, but, 42 days after the alloxan injection, gave a perfectly normal dextrose tolerance test. This animal, therefore, as a result of

TABLE II

BLOOD DEXTROSE TOLERANCE TESTS, BOTH ON ALLOXAN-INJECTED DOGS AND ON NORMAL CONTROLS; 1.5 GM. DEXTROSE PER KILOGRAM DISSOLVED IN 15 C.C. MILK PER KILOGRAM WERE ORALLY ADMINISTERED TO THE FASTING ANIMALS. BLOOD SUGAR WAS DETERMINED BEFORE THE ANIMALS DRANK THE MILK WITH DEXTROSE AND AFTERWARDS, AT THE END OF THE SPECIFIED LAPSES OF TIME

DOG NUMBER:	1	2	2	3	6
Condition of the dog:	Injected	Injected	Injected	Normal control, to be injected, later	Normal control
Milligrams of alloxan intravenously injected, per kilogram:	100	100	100		
Time after injection of alloxan:	8 days	5 days	42 days		
Before:	492	103	88	99	90
30 minutes	550	220	135	122	149
1 hour	605	220	134	148	148
2 hours	575	110	89	148	100
3 hours	480	105	87	93	82

TABLE III

MAIN COURSE OF EVENTS OF EXPERIMENTS WITH DOG 1

DATE (1943)	WEIGHT, KG. (FASTING)	FASTING BLOOD SUGAR	GLYCOSURIA		KETOSIS	REMARKS
			FASTING	AFTER FOOD		
9/2	13.0	102	no	no	no	Intravenous injection of alloxan, 100 mg. per kilogram in three portions; 15 minute intervals.
3		343	+++	++++	no	Normal-looking; very marked polydipsia and polyuria.
4	13.7	341	+++	++++	no	Rather depressed at moments.
5						Whole-day fast.
6	12.8	215				Normal-looking.
8	13.5	350				
9		492	++++	++++	++	Very marked lipemia. Dextrose tolerance test.
10	13.2					Vomits.
14				++++	++	Quite normal-looking.
18	11.7					
20	11.2					
22						Whole-day fast.
23	10.7	358	+	+++	+++	Laparotomy, under nembutal. Pancreatic biopsy.
24			+	+	+	Extremely depressed. Drinks and urinates very little. Up to now polydipsia and polyuria had been very marked.
25						Dies.

alloxan action, developed a very mild but unmistakable diabetes, and this was subsequently cured. I think that this fact is one of great interest.

*Varying Degrees of Severity of the Diabetes Produced by Alloxan.*—The diabetes of Dog 2 was, as we have seen, extremely mild; that of Dog 1 was of a rather severe character; it cannot be said that it was extremely severe, since no insulin was given and no food restriction was instituted, but there is no doubt that it was severe, as made evident by the dextrose tolerance test (Table II), as well as by the severity of the symptoms (Table III), including ketosis and marked lipemia. The diabetes of Dog 5 (Table VI) was a little less severe than that of Dog 1, but much more so than that of Dog 2. It is not possible to

TABLE IV  
MAIN COURSE OF EVENTS OF EXPERIMENT WITH DOG 2

DATE (1943)	WEIGHT, KG. (FASTING)	FASTING BLOOD SUGAR	GLYCOSURIA		KETOSIS	REMARKS
			FASTING	AFTER FOOD		
9/4	6.5	105	no	no	no	Alloxan injected, 100 mg. per kilogram intravenously; two portions, 5 minute interval.
5		125	no	+	no	
6	6.3	136	no	++	no	
8	6.5	120	no	no	no	
9		103	no	no	no	Dextrose tolerance test.
13			no	+	no	
14			no	no	no	From this day on, instead of former mixed unmeasured food, beef lung, 400 Gm. daily.
20	7.0		no	no	no	
10/14	7.5		no	no	no	
16	7.2	88	no	no	no	Second dextrose tolerance test

TABLE V  
MAIN COURSE OF EVENTS OF EXPERIMENT WITH DOG 3. THE OLD AGE OF THE ANIMAL SHOULD BE TAKEN INTO ACCOUNT WHEN JUDGING THE DEXTROSE TOLERANCE TEST (SEE TABLE II)

TIME	BLOOD SUGAR	REMARKS
9/8/43		
11:45 A.M.:	93	Last blood sample of dextrose tolerance test. Immediately afterwards, injection of first of three portions of a total dose of 300 mg. per kilogram of alloxan intravenously.
11:58 A.M.:		Injection of second portion.
12:15 P.M.:		Injection of third portion.
12:15 P.M.:	90	
4:00 P.M.:		Very depressed. While able to walk, prefers to lie down.
4:15 P.M.:	220	Paresis of the extremities, specially the anterior. The animal is thought to be hypoglycemic.
5:00 P.M.:		Semicomatose, emesis; a little later 5 Gm. dextrose are given per os. (The result of the blood sugar determination was not known until next morning.)
6:00 P.M.:		Ten grams dextrose intravenously; the condition of the animal improves considerably.
From 6:15 P.M. on:		Drinks water several times and each time vomits; falls again into a semicomatose condition.
7:45 P.M.:		Ten grams dextrose intravenously; great improvement.
9:30 P.M.:		Still in a comparatively fair condition. Ten grams dextrose subcutaneously, to prevent chances of further development of hypoglycemia.
Some time during the night:		Dies.

TABLE VI

## MAIN COURSE OF EVENTS OF EXPERIMENT WITH DOG 5

DATE (1943)	WEIGHT, KG. (FASTING)	FASTING BLOOD SUGAR	GLYCOSURIA	KETOSIS	REMARKS
9/11	14.8	110	no	no	Subcutaneous injection of alloxan; 75 mg. per kilogram.
13			+	no	
14			no	no	
15			no	no	Second subcutaneous injection of alloxan; 75 mg. per kilogram.
16, 17			no	no	
18		121	no	no	A subcutaneous induration in the site of the last injection is noticed. No tenderness. No signs of pain or discomfort. In- travenous injection of alloxan; 200 mg. per kilogram, in two portions; 20 minute interval.
19		308	+++	no	Polydipsia and polyuria.
20	14.5	281	+++	+	The induration has softened; seems to be an abscess and to contain gas. Still no tender- ness.
21					Animal seems to be very ill; eats very little.
22			+++	+	Liquid stools. Abscess breaks. Animal dies.

TABLE VII

EARLY REACTIONS OTHER THAN BLOOD SUGAR CHANGES ELICITED IN SOME DOGS BY THE  
INTRAVENOUS INJECTION OF ALLOXAN

DOG NUMBER:	1	3	4
Milligrams of al- loxan intraven- ously injected per kilogram:	100	300	300
Number of por- tions in which total dose was distributed:	3	3	1
Time intervals be- tween injec- tions:	15 minutes	15 minutes	
Time of appear- ance and de- scription of re- actions:	10 minutes after second and 5 minutes before third injection; eme- sis. From 30 minutes until 2½ hours after the last injection, the animal is slightly de- pressed, lying down most of this time, al- though being able to walk. From the end of 2½ hours after completion of the in- jection, the animal looks and behaves quite normal.	3¾ hours after completion of injection the animal is de- pressed and lying down, al- though able to walk. 4 hours id. id., paresis of extremities, especially anterior, and seems to be hypoglycemic, although blood sugar is 220. Five hours id. id., lying down, semicomatose. 5¼ hours id. id., emesis; condition im- proved by dextrose adminis- tration. 6½ hours id. id., re- peated emesis after drinking water; becomes worse and again improves after dex- trose. 9¼ hours id. id.; fairly good condition. Some time later, dies.	Immediately aft- er the injec- tion: emesis; extremely de- pressed. Dies 25 minutes after comple- tion of injec- tion.

say whether the observed degree of severity of the disease in Dogs 1 and 5 would have remained more or less unchanged had the animals remained alive. This is an extremely important point that remains to be elucidated by further research. If the severity of alloxan diabetes is, in some cases at least, of a reasonably stable degree, I think that the possibility of producing diabetes in a wide range of varying degrees of severity may prove a fact of great importance.

*The Early Blood Sugar Changes Elicited by Alloxan in the Dog.*—The fact that in three of five alloxan-injected dogs that lived long enough to become diabetic there was no need to give them any special protection against hypoglycemia in order to keep them alive is in sharp contrast with the death in hypoglycemia of nearly all the rabbits in Dunn and his co-workers' experiments<sup>2</sup> and with the strenuous fight against hypoglycemia that Bailey and Bailey had to carry on in order to keep their rabbits alive. The figures in Table I show that, although in dogs after alloxan there is, in general, a certain fall of the blood sugar level, this fall appears much later and is much less marked than in rabbits. None of the figures found in any of the dogs in this series may be called hypoglycemic. So far as the initial hyperglycemia is concerned, a typical and constant feature of the rabbit reaction to alloxan, only in Dogs 1 and 3 did I find a comparatively early rise of the blood sugar level. It is true that my blood sugar determinations were not frequent enough to make it certain that there were no other high figures. In any event there can be no doubt that, in the dog, the initial hyperglycemia, produced by alloxan, when it exists, begins later than in the rabbit and, on the other hand, lasts longer, and there is, at least, some reason to believe that the initial alloxan hyperglycemia in the dog, as a rule, is much less marked than in the rabbit.

*Reactions Other Than Blood Sugar Changes.*—Except for the very early death of some of Dunn and his co-workers' animals,<sup>2</sup> from all that has been published about rabbits it might be gathered that the reactions described by both Dunn and his co-workers<sup>2</sup> and Bailey and Bailey<sup>1</sup> are merely those associated with hypoglycemia. Brunschwig and his co-workers<sup>2, 3</sup> do not mention any reactions in their dogs, except early death, but in human subjects on which they have used alloxan at very high doses, reactions such as nausea, emesis, chilling, and icterus are mentioned. In three out of five dogs of the present series, reactions other than those generally associated with blood sugar changes were observed (Table VII). In Dog 1, emesis was elicited by only 66 mg. alloxan per kilogram, and after the 100 mg. per kilogram dose was completed, there was a short period of slight depression, the whole thing being in contrast with the total absence of any visible reaction in Dog 5 (Table VI) after a dose of 200 mg. per kilogram. Dog 3 showed a very severe reaction following a dose of 300 mg. per kilogram. Of special interest is the fact that, at a moment when the blood sugar was 220, I mistook the condition of the animal for typical hypoglycemia, and my erroneous conviction was reinforced by the fact that dextrose administration repeatedly improved the condition of the animal. Although no further blood sugar determinations were made and the animal died during the night, while not being watched, I am convinced that this dog's death was not due to hypoglycemia, as certainly was not that of Dog 4.

*Wide Variation of Individual Reactiveness to Alloxan.*—The same dose of 100 mg. per kilogram produced a severe diabetes in Dog 1 and a very mild and curable one in Dog 2. This is in agreement with facts described in rabbits by previous observers. Brunshwig, et al.<sup>3</sup> state that 200 mg. per kilogram are invariably fatal to dogs, but Dog 5 of the present series (Table VI) stood that dose without the slightest untoward reaction and developed a moderately severe diabetes; although it died within a few days, its death cannot be ascribed to a toxic action of alloxan.

*Pancreatic Changes.*—These will be extensively studied somewhere else. Suffice it to say here that, in a general way, our findings are in agreement with those of previous observers, and that in the pancreas specimen taken by biopsy from Dog 2, when it was unquestionably diabetic although in a very mild degree, no islet or other lesions were found. This shows that a noxious agent can elicit a diabetic functional disturbance of the endocrine pancreas even when no associated anatomic lesion may be detected, a point of interest in connection with problems of human diabetes, and in agreement with some observations of Brunshwig, et al.<sup>3</sup> on human subjects.

*Theoretical Considerations.*—When commenting about their discovery of the alloxan necrosis of the islands of Langerhans, Dunn, Sheehan, and Mc-Letchie<sup>5</sup> made some very interesting suggestions. One of them was that the islet necrosis might be caused by unbearable strain from overstimulation; the primary action of alloxan would be to overstimulate the islets to produce insulin, and what in their experiments was the final hypoglycemia might be taken for evidence of that overstimulation. This hypothesis is hard to conciliate with the fact that, as a result of alloxan, dogs may, as 1 and 5 of this series, develop destructive lesions of their pancreatic islets and a severe diabetes without having, at any moment, shown either blood sugar figures or external signs of hypoglycemia. It seems more probable that the noxious action of alloxan upon the cells of the islands of Langerhans should be of a more direct and, so to say, brutal character.

#### SUMMARY

1. The production of pancreatic diabetes in dogs by alloxan is confirmed.
2. Alloxan can produce diabetes of varying degrees of severity.
3. The diabetes produced by alloxan, when of a very mild degree of severity, may be spontaneously curable.
4. The initial blood sugar changes brought about in the dog by diabetogenic doses of alloxan are slower in their appearance and smaller in magnitude than those in the rabbit; the initial hyperglycemia begins later; when detected, lasts longer; and the figures found in the present experiments have been, with one exception, much lower. The subsequent fall begins much later and, in the present experiments, has never been found to reach hypoglycemic levels. None of the three dogs that became diabetic required any antihypoglycemic treatment in order to be kept alive.
5. In all the three dogs that survived the injection of alloxan for more than one day, diabetes, with hyperglycemia and glycosuria, was established twenty-four hours after the injection.

6. Toxic reactions, other than those related to blood sugar changes, such as emesis, depression, paresis, and torpor, have been observed in some cases. Two dogs injected with 300 mg. per kilogram died in a very short period of time. In one of these, a condition that could be mistaken for hypoglycemia existed at a moment when actually there was hyperglycemia.

7. There is a wide range of variation as to the individual reactivity of dogs both to the toxic and to the diabetogenic action of alloxan.

8. The hypothesis that the primary action of diabetogenic doses of alloxan on the islands of Langerhans might be one of overstimulation, resulting in temporal hypoglycemia from hypersecretion of insulin, is hard to conciliate with some of the presented facts.

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# LABORATORY METHODS

## GENERAL

### LABORATORY AIDS IN THE DIAGNOSIS OF MALARIA\*

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**M**ALARIA is a disease of prime importance in the tropics and may soon attain this distinction in regions in the United States where it was rare before the war. In this paper we wish to describe the methods which have been used with success in making the laboratory diagnosis of malaria in an army hospital operating in the South and Southwest Pacific theatre of war. The first year of practicing jungle medicine was a trial and error period for those of us not previously expert in the laboratory diagnosis of tropical diseases. Now that this adjustment is over, we feel that we are having good results in finding malarial parasites in soldiers suspected of having the disease. This has been accomplished, in some instances, by the use of standard techniques in staining blood smears, but as a rule, we have had to alter textbook procedures to meet jungle conditions. We have learned the techniques used by New Zealand and Australian medical officers, and we have developed certain modifications of our own. It is hoped that the recording of these may contribute something to the new history of malaria that is now being written throughout the world.

#### CONSIDERATIONS IN THE LABORATORY DIAGNOSIS

The finding of parasites, pigmented leucocytes, and a high percentage of monocytes in the blood smear are the outstanding considerations in making a laboratory diagnosis of malaria. However, a positive diagnosis of malaria should not be made unless parasites are found in the blood smear. It is difficult to find parasites in soldiers who are receiving suppressive antimalarial therapy, even when they have typical symptoms of malaria. Atabrine has so altered the picture that in less than 40 per cent will parasites be found in the initial smear, and, in these, many parasites will be atypical in appearance. In troops operating in malarial districts it is essential to collect both thick and thin smears from every febrile patient. Ideally, blood films should be collected before any antimalarial therapy is started, but of course in soldiers who are receiving suppressive therapy, this is impossible. If smears are repeated at intervals from four to eight hours, we have almost invariably found parasites before the sixth smear was taken. This is usually in the thick smear. It may

\*From the Laboratory—Medical Service of the Evacuation Hospital.  
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take several hours of searching to find even one parasite in the thin portion of the same slide.

It is the practice of this hospital to have thick-thin blood smears taken on patients suspected of having clinical malaria upon admission to the hospital. Treatment may be started even before the smear is examined if the patient is quite ill. It is our impression that the number of parasites is not noticeably diminished during the first twenty-four hours by the low dosage atabrine therapy previously in use. However, in those who receive quinine or in those receiving massive doses of atabrine it may be impossible to find any parasites in the blood after twelve hours of therapy.

#### PREPARATION OF A "THICK-THIN" SMEAR

After some months of making smears too thick or too thin, we have developed a technique which works very well. A heavy mark is drawn, with a wax crayon, across a glass slide about three-fourths of an inch from one end. A finger is cleansed, dried, and pricked with a lancet. A drop of blood, about the size of a small pea, is placed in the smaller area of the slide. Utilizing the ball of the bleeding finger as a spreader, the drop is spread to the size of a five-cent piece and thin enough to see the hands of a watch through the film. A very thin film is spread at the opposite end of the slide. With the thick portion up, the slide is placed in a semivertical position to dry and the excess blood from the thick smear is allowed to drain to the crayon mark. The slide is air-dried in this position and under a screen if flies are about. In damp weather it is necessary to dry the slides in a dry hot air chamber, easily made from a tin can.

#### TECHNIQUE OF STAINING A "THICK-THIN" SMEAR

When a large number of blood examinations for malaria are performed daily it becomes necessary to develop a rapid technique for staining films. For ordinary diagnostic purposes we have adopted a rapid method of staining malarial parasites in thick smears as described by Field.<sup>1\*</sup> The thick smear is ready for staining as soon as it appears dry (Fig. 1). The film is dipped for one second in Solution A. It is immediately dipped in clean water for a few seconds or until the stain ceases to flow from the slide. It is then dipped in Solution B for one second. The stain is flushed off and then rinsed gently in clean water. It is dried in a vertical position. There may be slight variation in timing with different batches of stain. The smear should be stained immediately after drying for the best results.

\*Field's Stain, modified.

Solution A: 1. Dissolve 1.25 Gm. of methylene blue chloride and 5 Gm. of sodium phosphate dibasic, Merck, in 50 c.c. of distilled water.

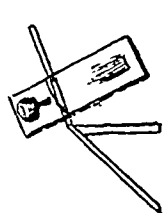
2. Bring to a boil. Then evaporate to dryness. (This is the most important step in preparing a good stain.)

3. Add 6.25 Gm. of potassium phosphate monobasic, Merck, dissolved in 500 c.c. of distilled water. Mix well and let stand for a few days. Filter before use.

Solution B: 1. Dissolve 5 Gm. of sodium phosphate dibasic, Merck, and 6.25 Gm. of potassium phosphate, Merck, in 500 c.c. of distilled water. Add Eosin Y, 1 Gm.

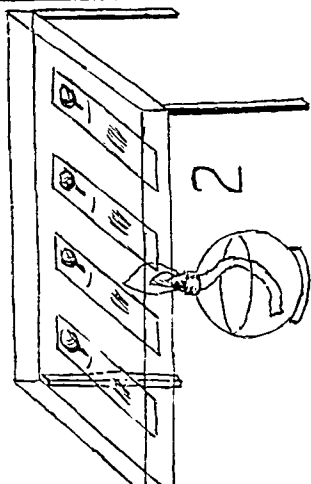
2. . . . . it becomes greenish. We have modified Field by omitting Azure B from mixture 1 this dye. We have compared our stain prepared for the Royal Australian medical laboratories of Australia. We are convinced that our stain compares favorably with the original in every respect.

# TECHNIQUE OF MAKING A "THICK-THIN" SMEAR FOR THE EXAMINATION OF MALARIAL PARASITES



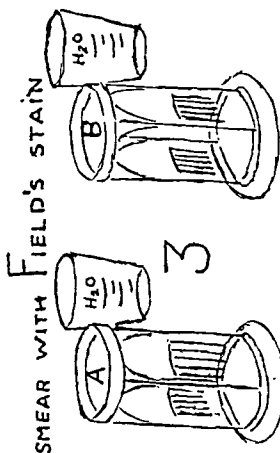
1

PROPER METHOD OF PREPARING  
"THICK-THIN" SMEAR



2

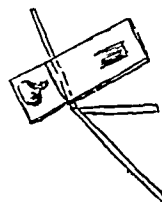
RAPID DRYING OF SLIDES IN DAMP  
WEATHER



3

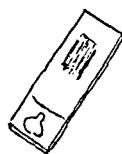
STAINING THICK PORTION OF  
SMEAR WITH FIELD'S STAIN

I. Dip thick smear in sol. A for 1 second.  
II. Rinse in water.  
III. Dip in solution B for 1 second, flush off.  
IV. Rinse in clear water



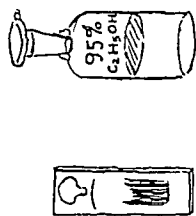
4

AIR DRY



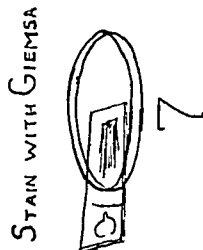
5

STAIN THIN PORTION OF  
SMEAR WITH WRIGHT'S  
STAIN



6

DECOLORIZE THIN PORTION  
OF SMEAR UNTIL IT IS A  
LIGHT PINK IN COLOR



7

STAIN WITH GIEMSA

STAIN 1:15 DILUTION FOR  
15-20 MINUTES. SLIDE IS  
PLACED FACE DOWN IN  
THE DILUTED STAIN. WASH  
AND DRY.



8

EXAMINE THICK-THIN SMEAR  
UNDER OIL IMMERSION.  
PARASITES ARE MOST NU-  
MEROUS AT THE POINT  
DESIGNATED ABOVE

After the thick portion of the smear is stained and dried, the thin smear is now ready for staining. We have found that a combination of Wright's<sup>2</sup> and Giemsa<sup>3</sup> stains gives nearly perfect results for examining parasites and red blood cells. Fourteen to sixteen drops of Wright's stain are floated in the center of the thin film. This is allowed to stand for one-half to one minute to fix the stain. Then it is diluted with twenty-four to twenty-six drops of buffer solution<sup>4\*</sup> or distilled water. The mixture is allowed to stand for from eight to twelve minutes. The slide is washed well with water. The smear is then decolorized with 95 per cent ethyl alcohol until it is a pale pink color. Freshly filtered stock Giemsa stain is diluted with fifteen parts of the phosphate buffer or distilled water and placed in a Petri dish. The thin portion of the smear is now placed face down in contact with the stain in the Petri dish for twenty to thirty minutes. It is then washed and dried.

Frequently it is difficult to get a perfect result with Wright's stain. Because of variation in batches of dye or because the technician may overstain the smear, the red blood cells appear too blue, making it difficult to find small trophozoites. The technique described helps correct this fault.

#### EXAMINATION OF THE "THICK-THIN" SMEAR

The appearance of the malarial parasites in the thick smear is very different from that in a thin smear. Whereas in the thin smear, the parasite with its typical red staining chromatin dot and blue cytoplasm is found in the infected red blood cell, in the thick smear the red blood cells are destroyed leaving the parasites free and concentrated. Under oil immersion and using the 5 $\times$  ocular, the thick smear is examined at the junction of the circumscribed portion and the fingerlike projection where the blood has drained toward the center of the slide. This portion is clearly seen when the slide is placed on a white background. It appears as a thin wavy brown-red line. This is not only the area of maximum dehemoglobinization, but it is at this point that the parasites tend to cling when the blood is dried with the slide in a semivertical position. The 5 $\times$  ocular is used initially because it is easier to look for parasites or pigmented leucocytes in a smaller field. In a smear which contains parasites and which is stained by Field's method, the chromatin dots stain a bright red to a crimson and can be seen easily. These dots are then examined with the 10 $\times$  ocular. In many instances the blue cytoplasm is found coexistent with the chromatin dots, confirming the impression that plasmodial parasites are present in the smear. In the thick smear parasites appear smaller and more shrunken than in a thin film.

*Plasmodium vivax* is characterized by a single red chromatin dot associated with blue cytoplasm. This has a tendency to assume many variations in form dependent on the stage of the parasite. Whereas in the young trophozoite the cytoplasm may appear as an "interrupted ring," in older trophozoites there is a decided tendency for the cytoplasm to disperse and arrange itself in clusters. In the presegmenting and segmenting schizonts the cytoplasm is more

\*This buffer is prepared by dissolving sodium phosphate dibasic, Merck, 9.47 Gm., and potassium phosphate monobasic, 9.08 Gm., in distilled water, making each solution up to 1000 c.c. The proportions of acid and alkaline phosphates may be adjusted to any pH. Usually for blood smears a pH of 7.0 is desired. This is made by mixing M/15 Na<sub>2</sub>HPO<sub>4</sub> 61.1 c.c. and M/15 KH<sub>2</sub>PO<sub>4</sub> 38.9 c.c. In the tropics we have had more success using pH of 6.4. This is made by mixing M/15 Na<sub>2</sub>HPO<sub>4</sub> 71.3 c.c. and M/15 KH<sub>2</sub>PO<sub>4</sub> 28.7 c.c.

compact and the chromatin divides into several irregular masses associated with cytoplasm. The schizonts are large and have yellow-brown pigment dispersed through the cytoplasm. Frequently this pigment is found free on the background of the thick film. Merozoites number from eight to eighteen. The microgamete is large with undivided red chromatin surrounded by a small amount of light-staining cytoplasm. This contains scattered red-brown granules and yellow pigment. Macrogametes cannot be distinguished from mature trophozoites.

The *Plasmodium falciparum* consists of a single or double red chromatin dot associated with a wisp of blue cytoplasm which resembles a comma or an exclamation point. Frequently scattered throughout a smear containing falciparum organisms are single red dots without any pigment or cytoplasm. In our experience the presence of these suggests that typical parasites will be found. The gametocyte of *Plasmodium falciparum* is the typical sausage- or crescent-shaped parasite which stains light blue and has a central golden-yellow mass of pigment. It is difficult to distinguish the sex in a thick smear. The young trophozoite and the gametocyte are usually the only forms of *Plasmodium falciparum* found in the blood. However, in very severe subtertian malaria, larger trophozoites and even schizonts may be present.

*Plasmodium malariae* is characterized by a chromatin dot which is larger than that found in the other plasmodia, a relatively small amount of dark blue cytoplasm which tends to form bands, and a large amount of coarse brown pigment which remains compact. The gametocytes resemble vivax but are smaller and heavily pigmented. *Plasmodium malariae* has been uncommon in our studies.

Difficulties arise when infections are light, as they frequently are in soldiers taking suppressive antimalarial therapy. Under these conditions parasites may appear degenerate and stain poorly.<sup>5</sup> The chromatin dot may stain darkly or the cytoplasm may be a very light or unevenly stained blue. They may be easily confused with blood platelets or reticular debris. However, in our experience, one can usually find at least two typical parasites in the smear.

In the thick smear the polymorphonuclear leucocytes stain violet-blue, with fine brownish granules. If pigment is present, it is usually gathered at one end of the cell and is yellow-brown in appearance. Monocytes stain deep blue and basophiles are recognized by predominating dark blue-black granules. The granules of eosinophiles stain a flat brown-red which are darker and larger than chromatin dots. Field<sup>6</sup> in his original article gives an excellent description of the appearance of the malarial parasites in the thick smear, and in addition presents some photographs of typical and atypical forms of plasmodia.

Early ring forms of *Plasmodium vivax* are difficult to distinguish from the trophozoites of *Plasmodium falciparum*. This is especially true in mixed infections. The differentiation in diagnosis is assisted by the fact that the young trophozoites of *Plasmodium vivax* are nearly always associated with older forms of the parasite. *Plasmodium falciparum*, on the other hand, are more consistent in size and shape. A smear repeated in twelve hours may clarify the diagnosis by presenting definite schizonts of *Plasmodium vivax*. However, as a rule, a thin smear is necessary to determine the type of plasmodium. The thin

smear should be prepared thin enough to compare the individual red blood cells with each other and in such a manner that there is a well-defined edge parallel to and about one-eighth of an inch from the edge of the slide. Large parasitic forms, like polymorphonuclears, tend to cling to the edge. Then one can usually differentiate the fine delicate ring of the falciparum from that of the coarser vivax which is found in enlarged red blood cells.

#### OTHER LABORATORY AIDS

It is our experience that the total leucocyte count in malaria is between 5,000 and 10,000 cells. Before a chill the leucocyte count tends to be lowest, while during the chill or shortly thereafter there is an increase in the number of cells, particularly monocytes. Twenty per cent of the cells may contain malarial pigment. The presence of pigmented monocytes and polymorphonuclear leucocytes are almost as diagnostic of malaria as the parasites themselves.<sup>7</sup>

Anemia is a characteristic feature of malaria. In recurrent cases, particularly, it is not infrequent to obtain a fall of from one to two million red blood cells and a 25 per cent drop in hemoglobin. In severe cases of malignant tertian malaria, a hemolytic anemia may be encountered with a high icterus index and increased urobilinogen in the urine or feces. All patients with cerebral malaria should be blood-typed upon admission, and a blood transfusion should be given at the first indication of rapid blood destruction.

When large amounts of atabrine are present in the urine, a bright yellow color results. However, a better method to determine if atabrine is present in the urine is to concentrate the atabrine by extraction with amyl alcohol by the method described by Wats and Ghosh.<sup>8</sup> It has been modified to facilitate interpretation, and as a matter of economy. Ten cubic centimeters of urine are alkalized by adding 1 c.c. of saturated potassium or sodium carbonate. One cubic centimeter of amyl alcohol is added to the solution and shaken well. It is permitted to stand until the alcohol layer separates. If atabrine is present, a yellow-green fluorescence can be seen in the alcoholic layer if examined in the sunlight. Ether may be substituted for amyl alcohol, but the yellow color is less intense. This test is of value in determining whether the patient has been taking suppressive antimalarial therapy prior to treatment, or, in those patients who have never taken atabrine prior to treatment, whether absorption and excretion is taking place. Similarly the urine can be tested for quinine.<sup>9</sup>

#### CONCLUSION

The laboratory diagnosis of malaria is not difficult. It is a question of good staining technique and examining the blood for parasites at the proper time. The rapid staining technique of thick smears as devised by Field is, in our opinion, ideal for rapid diagnosis and when a large number of smears must be examined as quickly as possible. Although many authorities feel that blood films should be dried in the horizontal position, in our opinion, when Field's stain is used, there is a definite advantage in drying the smear in the semi-vertical position. The parasites tend to concentrate in one area; this diminishes orientation time and facilitates examination. As far as we can determine, there are two disadvantages to Field's technique. First, if the slide is not washed

well, some eosinophilic staining debris clings to the slide. The examiner soon learns to differentiate these artifacts from parasites. Second, the stain fades, making it impossible to keep slides for permanent record. It is imperative that the thick smear be not too thick and that the smear is examined at the point where the parasites tend to concentrate. The standard or Wright-Giemsa thick smear staining techniques as described by Wilcox and Logan<sup>10</sup> are excellent, but are not as convenient as the technique described above. In a Giemsa-stained thick smear the chromatin dot is not as vivid, but the cytoplasm is more intense. Although it takes more time to stain a thin smear with the Wright-Giemsa combination than with a simple Wright's stain, it is well worth the effort in those instances where it is difficult to find a parasite or identify the type of plasmodia present. If the slide is stained face down in a Petri dish, the debris and sediment will fall off and a clean blood film will result.

#### SUMMARY

1. The laboratory routine used in the diagnosis of malaria which has been performed for over a year by a large army hospital in the South and South-west Pacific theatre of war is described.

2. A rapid technique for staining malarial parasites in thick smears, which has received little attention in the literature, is described. Similarly, a staining technique in thin smears, which has proved very satisfactory, is described.

3. In order to diagnose and treat patients with malaria properly, it is necessary to have confirmation of clinical diagnosis of malaria.

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# A DEVICE FOR THE PROTECTION OF MICROTOME SECTIONS\*

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**B**ANDS of microtome sections are usually deposited on a sheet of paper as they come from the knife, before being mounted on slides. This practice exposes the sections to the risk of being disturbed or scattered by the slightest draught. For better protection of the sections I recommend the following device which has been in use in our institute for some time.

The device consists essentially of a rectangular framework containing the sheet of paper on which the bands will be deposited, and of a glass cover sliding on the framework.

A wooden board bearing on its longer sides two ledges, transverse section shown in *B* of Fig. 1, serves as a support for the sheet of paper on which the bands will be deposited. A glass cover (3), indicated by dotted line, with a handle (4) is fitted so as to slide easily on the ledges. There are two shorter ledges (5) at both sides of the paper flush with the lower level of the long ledges.

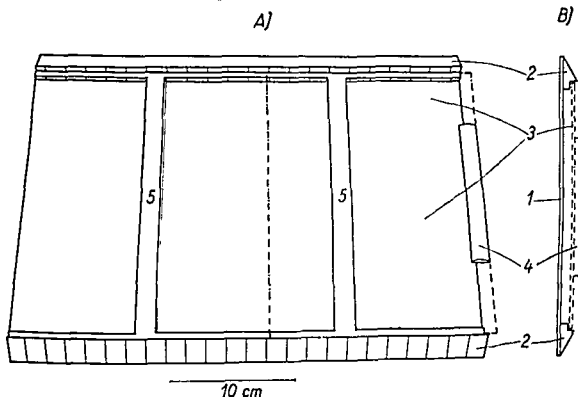


Fig. 1.

The series are deposited from the knife onto the paper in the framework. The glass cover is pulled by the handle from left to right, successively covering each new band. The framework protects the bands from any dust or draught which might come from the sides, while the glass affords protection from above. When we mount the sections we pull the glass cover to the right, successively uncovering them.

Since with this device we shift the glass instead of lifting it, we avoid eddies which might disturb the bands.

The size of the whole board was  $27 \times 38$  cm., to fit a sheet of  $14 \times 22$  cm. writing paper. The glass cover should slide on ledges at least 7 mm. high. The use of ready-made ledges as used for picture frames is most advisable.

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# A TIME AND TEMPERATURE GUIDE FOR INSPISSATING CULTURE MEDIA\*

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FOR many types of culture media which are prepared by inspissation, it is desirable to raise the temperature of the inspissator at a rather slow and steady rate in order to get a uniform product, free from holes and defects. One method to accomplish this is to make a chart of time and the corresponding temperature, and to have a clock which is turned back to the "zero hour" at the start of each inspissation.

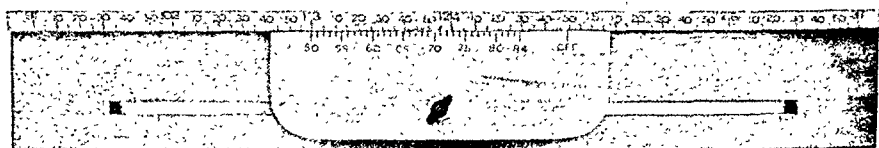


Fig. 1.

Another method, and one which we prefer, is to make a simple "slide rule," by which the temperature at the start of inspissation is set opposite the actual time of the day, and which indicates from then on the temperature which the inspissator should have at any time until the batch is complete. We use this temperature guide in making Petraghani's medium for cultivating tubercle bacilli; the temperature is raised from 50° to 84° C. during 1½ hours, and then held for twenty minutes at 84°.

The illustration shows the rule marked for this purpose. Time is marked on the long fixed scale, from 9 A.M. to 3 P.M. and again from 1 to 7 P.M. The smaller moveable part, which can be placed and held at any setting by the slot and wing nut bolt, is marked for the temperature, and the space between the 50° and 84° marks corresponds to 1½ hours. The temperature guide, as we made it, is 50 cm. long and 8 cm. wide; obviously any desired size can be used and may be graduated for any rate of temperature rise.

\*From the Laboratory of Queen Alexandra Sanatorium.  
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THE REACTION OF *XENOPUS LAEVIS* DAUDIN  
(THE SOUTH AFRICAN CLAWED FROG)  
TO HUMAN PREGNANCY SERUM\*

A NEW TEST FOR PREGNANCY. A PRELIMINARY REPORT

SAMUEL S. ROSENFELD, M.D., AND VILMA W. ROSENFELD, R.N., NEW YORK, N. Y.

CLOSE association with Weisman influenced us in employing the female *Xenopus* Frog Test for pregnancy. We followed the technique published by Weisman, Snyder, and Coates,<sup>1-3</sup> using a urine concentrate as described by them.

The results obtained by us with the urine concentrate were very satisfactory. However, having observed the action of human pregnancy serum in the rat and in the rabbit (Rosenfeld, Lapan, and Baron<sup>4</sup>), we decided to experiment on this species of frog with human pregnancy serum.

MATERIALS AND METHODS

Mature female frogs, *Xenopus laevis*, were employed. They had previously been tested by us with injections of urine concentrates from known pregnant and nonpregnant women, and the reactions were true; i.e., all the pregnant urines caused the extrusion of eggs whereas the nonpregnant urines failed to do so.

Serum was obtained in the ordinary manner by drawing blood from a vein and centrifuging the same after a short settling period. The serum was now ready for injection. This was usually done a day after the blood was obtained, but in several instances because of the lack of frogs the serum was not used for periods ranging up to four weeks.

Our first effort was to ascertain whether human pregnancy serum could cause extrusion of frog eggs. Having a store of pooled human pregnancy serum, we commenced our experiments with this material. We injected the first frog with 1 c.c. of pooled pregnancy serum, and after 48 hours, the result was negative. We repeated this experiment on another frog, this time injecting 2 c.c. of pooled pregnancy serum followed by another injection of 2 c.c. of the same, after 14 hours. The result was positive 8 hours after the last injection. We then found that single injections of 3 and even 5 c.c. of pooled pregnancy serum gave negative reactions, but additional subsequent injections of only  $\frac{1}{2}$  to 1 c.c. into the same animal yielded positive results.

We continued injecting the pooled pregnancy serum and found that three injections of 0.5 c.c. at 3- to 4-hour intervals yielded the best and the quickest results. Two injections of 0.5 c.c. at 3- to 4-hour intervals would also yield positive results, but the animals extruded many less eggs. Positive and satisfactory results were also obtainable with three injections of 0.25 c.c. No

\*From the Department of Obstetrics and the Laboratories of the Jewish Memorial Hospital.  
Received for publication, Dec. 6, 1943.



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positive results were obtained with amounts of less than 0.25 c.c., even when three injections were made at 3- to 4-hour intervals.

We then tested individual sera from known cases of pregnancy, usually employing three injections of 0.5 c.c. at 4-hour intervals. In a few instances the test was positive after the second injection, and on one occasion after the first. In these cases no injections were given after the test was seen to be positive.

Sera were examined from women in the first, second, and third trimesters. The tests were all positive. The earliest positive appeared 1½ hours after injection, but in the main, the time of appearance of the eggs ran parallel to that observed when urine concentrates are employed, i.e., in from 6 to 18 hours

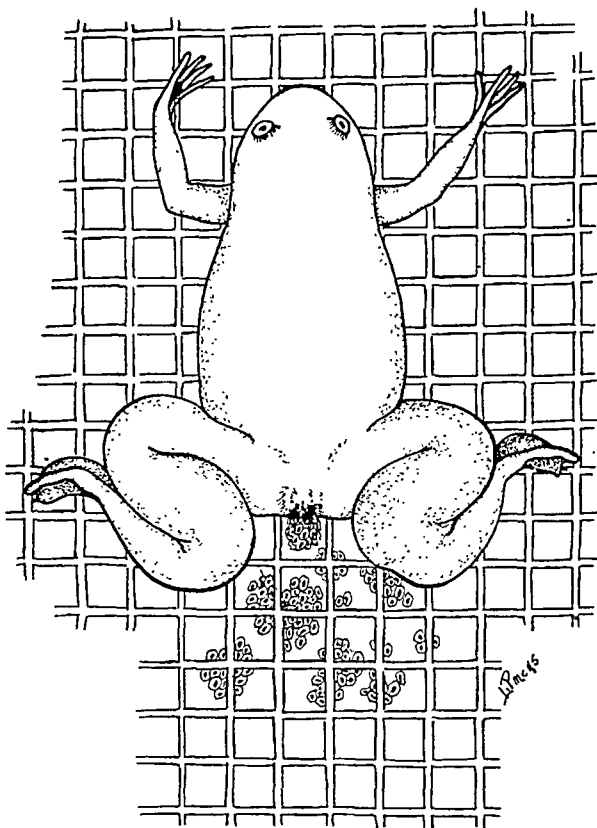


Fig. 1.—Positive test, actual drawing. Note eggs in the process of extrusion from cloaca.

For controls, male sera, definitely proved nonpregnant sera, and sera from women in the menopause were injected. All these sera gave negative results.

#### DISCUSSION

Hogben<sup>5</sup> showed that extracts of the anterior pituitary lobe caused external deposition of eggs in the hypophysectomized mature female *Xenopus laevis* (the South African clawed frog).

In 1934 both Shapiro and Zwarenstein<sup>6</sup> and Bellerby<sup>7</sup> working independently suggested the use of the *Xenopus* frog as a test for gonadotropic hormones in pregnancy urine.

Employment of serum has some advantages over the use of urine concentrate, and at certain times has definite indications. Specimens are easily obtained, and only a few c.c. of blood need be drawn, for 1.5 to 2 c.c. of serum is ample. After blood is drawn, it is a matter of minutes to get serum. The obtainment of the urine concentrate, however, necessitates several chemical procedures which in the best-equipped laboratories consume about three hours, and in the hands of the practitioner will take three or four times that.

Instances may arise where a test for pregnancy is clinically necessary, and yet it is desirable not to have the patient know or suspect the reason for performing the test. In such cases a serum test would arouse no suspicion, while a urine test in an intelligent woman would be almost a complete "give away."

While the time required to produce a positive reaction is about the same when either urine concentrate or serum is employed; nevertheless, since the first morning urine is usually used because of its higher hormone content, the actual or real elapsed time when serum is used is shorter because blood can be taken at any time of the day. Indeed, a positive test can be obtained by using serum before the urine specimen is even available for concentration.

*Summary.*—The South African clawed frog (*Xenopus laevis*) is a suitable animal for the demonstration of gonadotropic hormones. These hormones are present in the blood of pregnant women, and when injected into this species of frog cause an extrusion of eggs. The end result is macroscopic and can be read with great facility. Blood serum has several advantages, such as ease of obtainment and preparation; the "real" time for the appearance of a positive reaction is shorter, and in instances where it is desired to keep the patient from thinking that pregnancy is suspected, serum is preferable.

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## A VEHICLE FOR THE INTRAVENOUS ADMINISTRATION OF FAT-SOLUBLE HORMONES\*

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THE intravenous route for the administration of hormones is limited to those which are water-soluble. The fat-soluble hormones are usually restricted to some other method of parenteral injection. In experimental work it is often desirable to study the effects and the fate of fat-soluble hormones when given directly into the blood stream. This has been done by various workers by administering the hormone as a fine suspension in saline (Emery et al., 1943) or as a solution in alcohol (Cantarow et al., 1942). The danger of emboli formation in these procedures appears to be negligible when the amount of the hormone administered is small. However, the low solubility of some of the fat-soluble hormones, notably estrone, may require the use of relatively large amounts of the solvent. Unfortunately, because of their deleterious effects, the usual fat solvents cannot be administered by vein in large quantities with any great degree of safety.

Because of their properties, the suitability of the higher polyethylene glycols were studied by us, particular attention being paid to a compound known as "Carbowax 1500."† This compound is characterized by a large number of ether linkages and a high degree of chemical inertness. The molecular weight is about 1250, density 1.152<sup>20°</sup>, melting point between 30 and 33° C., and pH of 50 per cent aqueous solution 7.6 (glass electrode). At room temperature the substance is light amber in color with the consistency of petrolatum, while in the liquid state it is faintly brown and somewhat viscous. It is probably a mixture of several of the higher polyethylene glycols.

The polyethylene glycol is soluble in blood and blood serum in all proportions. Single intravenous injections of 50 mg. androsterone, 50 mg. testosterone, and 150 mg. desoxycorticosterone, each dissolved in 3 c.c. "Carbowax 1500," have been made in dogs without untoward reactions. While these hormones precipitate out from solution when the solution is added in vitro to blood serum, reactions due to intravascular precipitation appear to be negligible. The results of intravenous administration of these hormones will be presented at a later date; the present paper is a preliminary report on the toxicity of the polyglycol when given by vein.

The only toxicity studies on "Carbowax 1500" reported to date are those of Smythe et al. (1941, 1942). No microscopic abnormality of any of the organs examined was found in rats receiving the polyglycol in drinking water up to 4.05 Gm. per kilogram body weight per day for ninety days. Much larger single

\*From the Department of Physiology, Jefferson Medical College,  
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†We are indebted to Carbide and Carbon Chemical Corp., for a generous supply of the polyethylene glycols.

doses fed to albino rats, guinea pigs, and rabbits were likewise without demonstrable effects on the liver and kidneys.

We have administered "Carbowax 1500" intravenously to twelve rabbits in single doses of 1 to 2 Gm. per kilogram body weight without observing any reactions. Four male mongrel dogs were given each by vein 0.5 Gm. per kilogram of the polyglycol and no disturbance was noted in respiration, pulse, or temperature during the period of four hours following. All dogs were still in apparent good health two months later.

Three female dogs and one male dog were given each 0.75 Gm. "Carbowax 1500" per kilogram per day for three successive days and blood studies were carried out for a period of three weeks. The red and white count, hemoglobin, and hematocrit remained normal throughout this period. Three of these dogs were sacrificed one, four, and six months later without any demonstrable pathologic changes found. The fourth dog, a female, is still living and in good health twenty months later. The Carbowax injections in this dog did not interfere with the birth of five puppies six months after administration.

The effects of the polyglycol on the blood pressure was studied in nine dogs under pentobarbital anesthesia and on six unanesthetized rabbits. When administered intravenously in 50 per cent solution in distilled water, no effect was observed in the dog in doses up to 4 Gm. per kilogram. When administered undiluted, there was often a slow rise in blood pressure of about 15 mm. Hg; this returned to normal within 15 to 25 minutes. This hypertensive action was noted more frequently when higher doses (3 to 4 Gm. per kilogram) were used. Intravenous injection of 0.5 to 1.0 Gm. per kilogram of the undiluted polyglycol produced in the rabbit a transitory rise in blood pressure (measured in the central artery of the ear) of 5 to 15 mm. Hg.

These experiments do not indicate the limit of tolerance for the "Carbowax 1500" nor do they reveal the ultimate fate of the substance after administration. Better chemical tests for the identification of the substance must be developed for study of the latter problem. These preliminary results are presented at this time with the caution that further investigation is needed. We believe that not only "Carbowax 1500" but also other higher polyethylene glycols may prove suitable vehicles for the intravenous administration of fat-soluble hormones (and vitamins), at least in experimental work. The small amount of the hormones usually required to produce the desired physiologic effect and the relatively high solubility in the polyglycol are in favor of the procedure.

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# CULTIVATION AND PRESERVATION OF DIPLOCOCCUS PNEUMONIAE AND MYCOBACTERIUM TUBERCULOSIS IN MARKET EGGS

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THE recent publication by Blattner, Keys, and Hartmann (Archives of Pathology 36: 262, 1943) adds another contribution to the use of fertile eggs as culture medium for a number of delicate microorganisms. This method has come to the fore as a result of the work of Goodpasture et al., by which the chick embryo has been established as a standard medium for most of the pathogenic viruses.

It should, however, be emphasized that the value of fertile eggs as a nutrient medium for bacteria has been stated exactly one-half a century ago by Maffucci\* (1894). Even earlier (1888), Hueppe† reported that "the sterile unboiled egg is an excellent medium for many microorganisms." Therefore, it seems strange that while nowadays the hatching egg has come into vogue as a medium for many pathogens (including certain spirochetes), the use of the ordinary market eggs, in spite of the obvious advantages, has not been resumed to any extent. Furthermore, it must be considered that inoculation of infective material into fertilized eggs does not result in a true cultivation of the implanted microbes; it is rather an infection of the developing embryo, which, including its membranes, is being invaded by the microorganisms, causing death of the embryo in a short time.

On the other hand, the common fresh market egg has proved to be a very suitable medium for pathogens. This is demonstrated by the following study for which we chose diplococcus pneumoniae and mycobacterium tuberculosis. The technique employed was as follows:

The natural air space end of the egg was painted with tincture of iodine, and a small hole was drilled into the shell with a thin sterile burr. A few drops of blood from a mouse infected with pneumococcus Type I and III respectively, or a few drops from a broth culture, or of a saline suspension from a plate culture were injected. After sealing the opening with sodium silicate, the eggs were incubated either at room temperature or in an electric incubator at 100° F.

It was found that the growth of pneumococci in market eggs *proceeded at room temperature to the same extent as in the incubator*. By aspirating with a sterile syringe, specimens were taken at three-day intervals from different areas of the eggs. Examination of stained smears revealed that the pneumococci in pure culture were spreading throughout the eggs.

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\*Maffucci, A.: *Centralbl. f. allg. Path. u. path. Anat.* 5: 1, 1894.

†Hueppe, Ferd.: *Centralbl. f. Bakt.* 4: 30, 1888.



Transmission to animals was successful with material from eggs up to eight weeks after inoculation, without loss of virulence or type specificity. Egg material, yolk as well as white, in a dilution of 1:1,000,000, when injected intraperitoneally into mice, caused death of the animals within eighteen to twenty-four hours. The virulence of the cultivated strains, as tested in mice and rabbits, remained unaltered after repeated egg passages or prolonged cultivation in the same egg. The Neufeld capsule swelling test was applied to yolk and egg white material, washed with saline to remove adhering protein substances. The results indicated that the type specificity was maintained.

The tubercle bacillus (*typus humanus*) could be cultivated in the same manner without difficulties. The strain used had been recently isolated from an active case. After one transfer on Petroff's medium, culture material was inoculated into eggs. In fertile eggs, growth of the bacillus was found to be almost limited to the chorio-allantoic membrane. In ordinary market eggs, however, development of the bacilli is not confined to isolated parts but spreads throughout the egg. By this technique fair multiplication of the bacillus is being obtained on the fifth or sixth day following inoculation. This is the case whether the eggs are kept in the incubator or at room temperature.

Microscopic examination of stained smears from aspirated egg content shows bacilli arranged in irregular groups, sometimes in clumplike aggregates. By intraperitoneal injection in guinea pigs it was shown that the virulence of the cultivated bacilli did not differ from that of the original culture.

These observations prove that the ordinary fresh market egg is a suitable and valuable medium for the cultivation and preservation of pneumococci and tubercle bacilli. It can just as well be utilized for the primary isolation of these microorganisms and probably for others as well. It is noteworthy that the author did not encounter any difficulty because of nonsterile eggs or secondary contamination. There is no need for using fertilized eggs. The normal market egg offers the advantage that it is by far the cheapest and simplest nutrient medium; it is easily obtainable and can be kept at room temperature. By using this method, the physician with restricted facilities is enabled to cultivate bacterial agents from suspected material (sputum, body fluids, excretions) and to perform the "typing" in cases of pneumonia. In doubtful tuberculous infections, cultivation of the Koch bacillus in eggs may prove an acceptable substitute for the animal experiment.

## ON THE USE OF MALLORY'S PHOSPHOTUNGSTIC ACID HEMATOXYLIN FOR STAINING INTESTINAL PROTOZOA\*

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AT THE present time, perhaps as never before, it is essential that diagnostic laboratories be prepared to make accurate fecal examinations for intestinal protozoa. A major handicap to such examinations, and in particular to identification of *Endamoeba histolytica*, is the nature of the staining routines usually recommended for preparation of permanent slides.

The methods most commonly outlined in books on laboratory procedure require the use of Heidenhain's iron hematoxylin or one of its many modifications. Most of these involve overstaining of the specimen, to be followed by destaining. Because of the size of the organisms to be identified, differential destaining must be followed with the microscope and at high magnification. Except in experienced hands this process is uncertain and prolonged.

Procedures by which differential destaining may be omitted, either by carefully timed staining periods<sup>1</sup> or by acidifying the stain,<sup>2</sup> have not given satisfactory preparations in our experience.

A stain that seems to meet the needs of routine examinations rather completely is Mallory's phosphotungstic acid hematoxylin, used as indicated here. The original description<sup>3</sup> of the procedure by which this stain was applied to fecal smears does not give entirely satisfactory results: first, apparently because fixation time is too short; second, because the fixative advocated does not contain acetic acid; and third, because the staining period cannot be stated exactly.

In our hands the following schedule has been tested many times and has given satisfactory preparations regularly. Nuclear stains have permitted ready identification of intestinal amoebae and flagellates, although structural details may not be quite so sharply defined as with the best of iron hematoxylin preparations.

1. Prepare fecal smears in the usual way and fix in Schaudinn's fluid or in any of its modifications, which contain about 5 per cent acetic acid; 1 to 24 hours. (Bouin's fluid has not given useful preparations.)
2. Remove bichloride of mercury from smears in the usual way.
3. Wash in 70 per cent alcohol to remove iodine.
4. Wash in water.
5. Stain in Mallory's phosphotungstic acid hematoxylin for 20 to 30 minutes. The time interval will vary with the age of the stain or its degree of "ripeness" and must be determined by trial.

\*From the Penrose Research Laboratory, Zoological Society of Philadelphia and the Department of Pathology, University of Pennsylvania.  
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6. Wash in running water for five minutes or until the smears become blue.

7. Dehydrate slowly in 95 per cent and absolute alcohols. Otherwise smears may appear cloudy when examined with oil immersion lens.

Cytoplasm of the trophozoites of amoebae and flagellates appears pale blue and the nuclei darker blue, but within cysts, nuclei are much more clearly stained than usual, chiefly because the cytoplasm is so lightly colored. In smears containing cysts and trophozoites of several species of amoebae the larger specimens stain more deeply but all can be identified.

It is to be remembered, however, that no staining method can compensate for degenerative changes within nuclei of cysts and trophozoites of intestinal protozoa when stool specimens are held too long at room temperatures before smears are prepared. That such changes occur rapidly is understood too infrequently.

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## LABORATORY SUPPORTS\*

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IT IS not uncommon to find that the supports (ring stands) which are available in the laboratory are either of the wrong size or are found to be too light in weight, a fact especially noticeable in the performance of microanalytical work. We found that supports with various bases can be simply and inexpensively made in the laboratory. The bases are cast from melted scrap lead, using a plaster of Paris matrix or a discarded tin can as a mold. Scrap lead can be purchased for a few cents a pound from a junk dealer; the price of new lead is about eight cents.

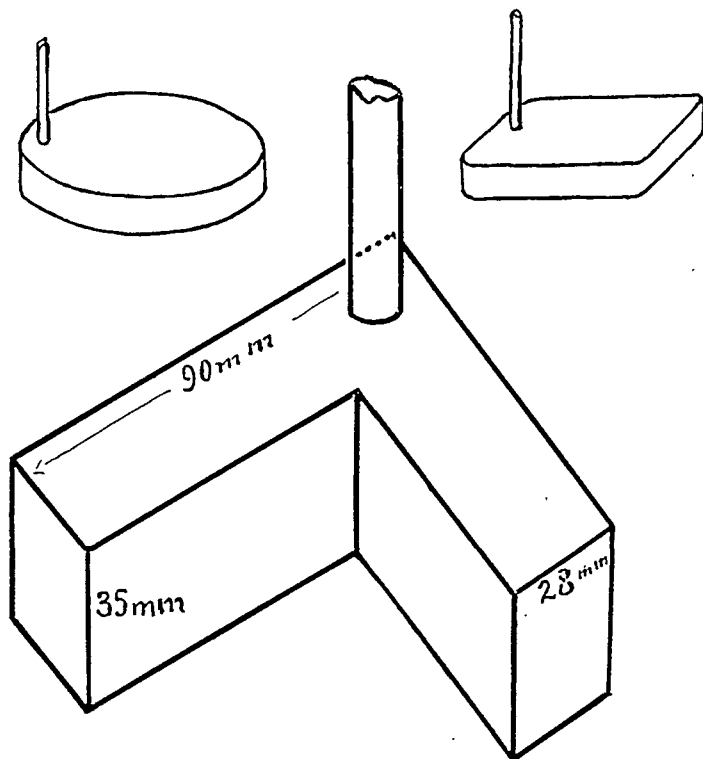


Fig.

The "patte" is made by nailing wood pieces together. The wood must be painted. The top is painted with plaster of Paris.

pieces of wood together. The top is painted with plaster of Paris about 1/4 inch thick.

\*From  
Received

of Biochemistry,  
Dec. 21, 1943.

ty Sch. fine.

one-half inch high is poured into the box first; the wood model is placed on top of this layer, and the rest of the plaster is poured around the pattern. After the mold has hardened, the wood is removed. It is advisable to drive two or three nails into the pattern to facilitate removal. The mold is dried in an oven or by any other suitable method.

Before the lead is poured, the support rod is put in position in the matrix, holding it fairly rigid and exactly vertical in a burette clamp. The rod should be notched with a file or a chisel in a number of places for better contact. We find that a so-called brass-plated curtain rod ( $\frac{3}{16}$  inch) serves very well for smaller stands. Short lengths of small pipe can also be used. The lead is melted, the dross is removed from the surface, and the metal is poured into the form. If the pattern was smooth, the stand can easily be removed and the mold used again.

Round and oblong small bases can be cast by using flat tin cans as molds. If the can is coated with library paste and allowed to dry, the lead will, as a rule, not stick to the form. Fig. 1 illustrates such stands. The L-shaped support ( $28 \times 35 \times 90$  mm.) weighed about three pounds. If desired, the corners can be rounded and the assembly painted.

## BLOOD AGAR PLATES\*

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ONE of the inconvenient problems of the small clinical laboratory is the question of a constant supply of sterile blood for blood agar plates commonly used especially for cultures of streptococci, pneumococci, gonococci, and for the hemophilic bacteria. This ordinarily involves preparation of glassware, defibrinating agents, and sterilization of syringes, not to mention the keeping and feeding of animals or the search for a willing human donor.

Practically all hospital or clinical laboratories receive blood specimens daily for serologic examinations. These are naturally collected aseptically and, if from local source, quickly reach the laboratory in an essentially sterile condition.

For many years we have followed the procedure of breaking loose the clot with a sterile loop, then placing these tubes in the refrigerator. When the clot has retracted, we draw off aseptically only the minimum amount of serum required for the given test. The tube containing the clot and residual serum is held in the refrigerator at about 6 to 7° C.

As the demand arises, we rotate such a tube of blood clot and serum rapidly between the hands. The serum takes up the free corpuscles and commonly yields sufficient dilute blood to make one or sometimes two blood agar plates rich enough in color and in serum to serve excellently both for enrichment and for detection of *alpha* or *beta* hemolysis.

If the blood is collected and handled aseptically, we have rarely a contamination in such plates. Since they are poured immediately before streaking, an occasional isolated contaminating colony is of little importance. It need scarcely be emphasized that these plates must be freshly prepared, lest minute colonies develop on standing and be distributed by the loop if inoculated on the following days.

It is well also to date the tubes and to use the most recent acquisition, and constantly to replace the older unused tubes with fresh stock.

By this simple expedient we have for years evaded the inconvenience commonly associated with this problem.

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\*From the Department of Medical Bacteriology and Public Hygiene, West Virginia University School of Medicine.  
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# THE EFFECT OF THE DISTAL APPLICATION OF A SPHYGMOMANOMETER CUFF ON LOCALIZED VENOUS PRESSURE AND AS AN AID IN VENIPUNCTURE

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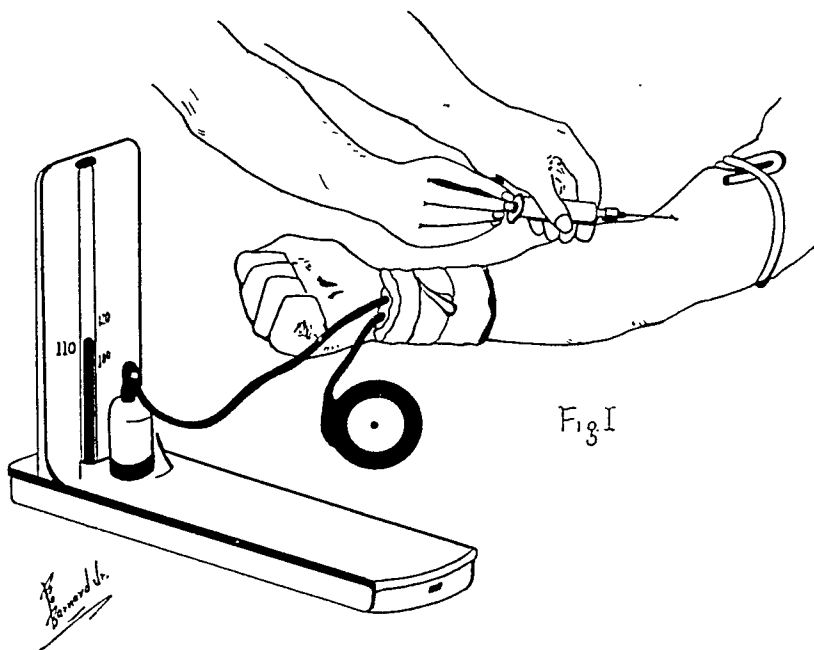
TO FACILITATE venipuncture, those expedients which result in a rise of venous pressure around the site of election are employed. Gunther<sup>1</sup> has stressed the role of intramuscular pressure in the maintenance of venous return flow and illustrates his point by citing the practically utilized turgidity of the antecubital veins of the proximally tourniqueted arm when the fist is clenched and the muscles of the forearm are thereby put under increased tension. He attributes the positive venous pressure in the extremities to a reflected positive intramuscular pressure. The author is inclined to believe that a major portion of the positive venous pressure in the superficial veins of the extremities is a reflection of the positive capillary pressure of the skin, citing, in this connection, that antecubital venipuncture is much more easily performed when the skin capillaries are not constricted by cold or by shocklike states.<sup>2</sup>

It is common knowledge among the physicians and nurses who staff the various blood donor centers that constriction around the wrist or pressure upon the flexor surface of the forearm will considerably augment the flow of blood from the site of the antecubital venipuncture. This phenomenon cannot be due, exclusively, to a "milking out" of stagnant blood in the forearm. In a series of experiments designed to elucidate the mechanism of this augmentation, it was found that steady pressure brought about by inflation of a blood pressure cuff, applied around the wrist, to an extent above that of the mean arterial pressure would, in most instances, result in a trebling of the rate of venous blood flow. Where the venipuncture had not already been performed, the superficial veins would become very prominent, turgid, and easily entered by the needle. In obese subjects the median cephalic vein, ordinarily undetectable after the usual application of the tourniquet, becomes easily distinguishable by palpation from the surrounding (and particularly in females, the frequently overlying) muscle and thereby becomes the vein of election over the median basilic for phlebotomy.

Fig. 1 illustrates the procedure. The cuff on the wrist is first applied; then the tourniquet, which consists of pliable rubber tubing about 1 inch in diameter, is tied *tightly* around the upper arm being secured by a single looped slip knot for ready release. The cuff is then inflated to from 100 to 120 mm. Hg. Either of the main antecubital or one of the superficial dermal veins will be easily accessible in over 99 per cent of subjects, however obese. Since it is undesirable to protract the double constriction, antiseptic preparations had preferably been made over a wide area around the antecubital fossa, beforehand.

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The procedure is not recommended for unmasking the veins of subjects in shock for the purpose of infusion. In fact, it should not be employed except on relatively sound individuals for the securing of blood for testing or for transfusion. These considerations arise from certain observations made in connection with its use. In the first place there is the possibility that the increased venous pressure is brought about by an increased capillary flow, the latter in turn being a reaction to trauma in the form of localized anoxia. For, if ordinary venous stasis from the proximal tourniquet were the only operative factor, it would be necessary to have the latter only sufficiently tight to exert a pressure slightly above that ordinarily extant in the veins of the extremities; in no physiologic instance greater than 20 mm. Hg. However, as in recent ordinary practice, there is substituted for the proximal tourniquet, a sphygmomanometer cuff; the latter must be inflated to above the diastolic arterial pressure before pronounced augmentation of the venous pressure, distal to it, results. In the described method, the proximal tourniquet is tied correspondingly tightly.



Secondly, the blood collected by this procedure shows a departure from the physiologic clotting sequence. This may be noticed in subjects in whom attempted venipuncture has caused injury to the vessel wall with the resultant formation of a hematoma. Usually such extravasation collapses the vein so that blood cannot be secured; if a flow does result, then clotting induced by extrahematic thrombokinase will shortly terminate it. These limitations do not hold with the tight proximal tourniquet. It is possible to continue the collection of blood in the presence of a large hematoma by the application of the described procedure, even if the needle is not within the vein and provided its lumen is not occluded, the supply being maintained from the hematoma, itself, which



constitutes the reservoir fed from the rent in the vein wall and in which reservoir the blood remains fluid much longer than would be the case under ordinary circumstances. This fact is cited, not in advocacy of the practice of collection of blood from hematomas (which may, however, be a useful expedient where the particular blood sample must be obtained), but to point out that there may be a physiologic alteration in the blood so collected and that the procedure is called for only in those instances where the ordinary ones do not suffice.

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## CHEMICAL

### THE RELATION BETWEEN THE PANDY TEST AND TOTAL PROTEIN CONTENT OF SPINAL FLUID\*

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SINCE the reports in the literature regarding the relationship between the Pandy test and the total protein in the spinal fluid are contradictory, and since it is the policy in some hospitals not to perform a quantitative protein estimation on the spinal fluid in the presence of a negative Pandy test, we decided to determine the relationship between the two.

TABLE I

	TOTAL	BELOW 50 MG.		ABOVE 50 MG.		ABOVE 100 MG.	
		NEG. PANDY	POS.	NEG. PANDY	POS.	NEG. PANDY	POS.
Brain tumor	153	48	5	46	54	0	20
Spinal tumor	55	19	1	14	21	0	13
Convulsive state	36	31	0	5	0	0	0
Vascular Disease C.N.S.	160	104	6	36	14	0	2
Degenerative Dis. C.N.S.	285	222	11	41	11	0	5
Infectious Dis. C.N.S. (except syphilis)	69	33	4	9	23	1	9
Traumatic Encephalopathy	25	19	1	5	0	0	0
Functional Nervous Disease	24	16	2	5	1	0	0
Peripheral Neuritis	33	17	0	9	7	0	4
Muscular Dystrophies	15	14	1	0	0	0	0
Syphilis	86						
Paresis		1	1	3	10	1	2
Tabes		14	1	6	1	1	0
C.N.S. Lues		24	4	12	9	1	3
Miscellaneous	59	38	1	14	6	9	4
Neurofibromatosis							
Arthritis							
Dysendocrinism							
Optic Atrophy—Unknown etiology							
Migraine							
Cerebral Agenesis, etc.							

The records of 1,000 neurologic admissions were examined. The nature of the clinical material used in this study is shown in Table I. Ling's<sup>3</sup> method was used for the protein determinations. It gives comparable results to the other procedures employed in estimating the total protein content. Although 37.9 mg. per 100 c.c. is given as the highest range of normal protein by this method, we consider values below 50 mg. per 100 c.c. as still being within normal limits. Table II indicates the distribution of the cases according to protein content and the results with the Pandy test. Table III summarizes our findings. It is interesting to note that 57 per cent of the cases had a negative Pandy

\*From the Neuropsychiatric Service, Montefiore Hospital, New York, S. P. Goodhart, Director.

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reaction and a total protein of 50 mg. per 100 c.c. or over. Above 100 mg. per 100 c.c., 17 per cent had a negative Pandy. The protein level at which the Pandy reaction became positive in more than 50 per cent of the cases was 70 to 75 mg. per 100 c.c.

A survey of the literature shows that many authors are of the opinion that a negative Pandy reaction definitely implies physiologic concentrations of protein and that consequently a negative Pandy reaction does not occur with pathologically increased protein concentration. Among the investigators who hold this view are Zaloziecki,<sup>14</sup> Greenfield,<sup>3</sup> and Eskuchen.<sup>2</sup> Liebold,<sup>7</sup> on the other hand, states that a positive Pandy may be found with a protein content between 16 to 30 mg. per 100 c.c. Izikowitz,<sup>4</sup> after studying 618 cases, concluded that negative Pandy reactions do not exclude the possibility of pathologically increased total protein and globulin concentration values. Positive reactions are no proof of pathologic protein increase nor do negative reactions constitute proof of physiologic protein relations. Neel<sup>10</sup> states that a quan-

TABLE II

PROTEIN CONTENT	TOTAL NUMBER CASES	NUMBER WITH POSITIVE PANDY	PER CENT WITH POSITIVE PANDY	NUMBER WITH NEGATIVE PANDY	PER CENT WITH NEGATIVE PANDY
5- 9.9	2			2	100
10- 14.9	4			4	100
15- 19.9	21	2	10	19	90
20- 24.9	61	1	2	60	98
25- 29.9	126	2	2	124	98
30- 34.9	142	6	6	136	94
35- 39.9	109	9	10	100	90
40- 44.9	84	7	9	77	91
45- 49.9	89	11	14	78	86
50- 54.9	68	9	14	59	86
55- 59.9	64	12	19	52	81
60- 64.9	59	17	34	33	66
65- 69.9	53	23	44	30	56
70- 74.9	19	10	53	9	47
75- 79.9	19	13	68	6	32
80- 84.9	4	3	75	1	25
85- 89.9	2	1	50	1	50
90- 94.9	3	3	100		
95- 99.9	7	6	86	1	14
100-199.9	50	37	74	13	26
200-299.9	9	9	100		
300-499.9	11	11	100		
500-1935	5	5	100		

TABLE III

THE RELATION BETWEEN THE PANDY AND TOTAL PROTEIN CONTENT OF SPINAL FLUID

	NUMBER CASES	PER CENT		NUMBER CASES	PER CENT
Total number with Positive Pandy	195	19.5	Total number with Negative Pandy	805	80.5
Number with Positive Pandy and Protein Below 50 mg. per 100 c.c.	38	6.0	Number with Negative Pandy and Protein Below 50 mg. per 100 c.c.	600	94.0
Number with Positive Pandy and Protein Above 50 mg. per 100 c.c.	157	43.0	Number with Negative Pandy and Protein Above 50 mg. per 100 c.c.	205	57.0
Number with Positive Pandy and Protein Above 100 mg. per 100 c.c.	62	83.0	Number with Negative Pandy and Protein Above 100 mg. per 100 c.c.	13	17.0

titative protein determination cannot be dispensed with even if the Pandy reaction is negative. Merritt and Fremont-Smith<sup>9</sup> write that the Pandy test is useful in indicating the presence of increased protein. However, they state that the "performance of a qualitative test does not give an accurate estimation of the degree of increase in the protein content." Quantitative tests should also be done. Dickson<sup>1</sup> noted that in infectious disease an increase in globulin can be detected when the total protein is as low as 30 mg. per 100 c.c.

The Pandy test is considered to be an index of the globulin fraction of the spinal fluid (Kafka,<sup>5</sup> Levinson<sup>6</sup>). In his paper, however, Pandy<sup>11</sup> stated that the test is not only for globulin but also for albumin. Schmitt,<sup>13</sup> and Merritt and Fremont-Smith<sup>9</sup> also believe it is not a definite test for globulin. Pappenheim<sup>12</sup> writes that with a negative Pandy reaction the total albumin content of the spinal fluid is always low and that a negative reaction excludes with certainty a pathologic increase of albumin. This, he maintains, holds except for isolated instances. An explanation for the negative Pandy with increased total protein may, therefore, be an increase of one or other of the fractions. Further work will evidently have to be done to clarify the relations of the globulin and albumin fractions to the Pandy test.

Our results as indicated confirm those investigators who maintain that the total protein may be increased despite a negative Pandy reaction. The level of protein at which more than 50 per cent of the cases in our series began to show a positive Pandy was 70 to 75 mg. per 100 c.c. We also found cases in which a positive Pandy was demonstrated with total protein content below 50 mg. per 100 c.c. and even as low as 20 mg. per 100 c.c.

#### SUMMARY

1. The records of 1,000 neurologic admissions were examined as to the relation between the Pandy test and the total protein content of the spinal fluid.
2. Of the cases, 59 per cent had a negative Pandy with a total protein content of 50 mg. per 100 c.c. or over.
3. Of the cases with a total protein content of 100 mg. per 100 c.c. or over, 83 per cent had a positive Pandy reaction.
4. The level of protein at which more than 50 per cent of the cases began to show a positive Pandy was 70 to 75 mg. per 100 c.c.
5. Our results indicate that a negative Pandy may be present with a total protein above 50 mg. per 100 c.c.

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# MEDICAL ILLUSTRATION

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## REPAIR OF CRANIAL DEFECTS BY CAST CHIP-BONE GRAFTS

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### A PRELIMINARY REPORT

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THE restoration of the contour in cranial and frontal bone defects was achieved during the first World War by one of three methods:

1. Costal cartilage grafts (Morestin<sup>1</sup>) were used to fill out the defect by a cartilaginous and fibrous tissue network.
2. Foreign materials, such as celluloid and silver plates, were employed. Many of these were later removed following foreign body reactions.
3. Osteoperiosteal grafts taken from the tibia were used extensively and successfully by Delangeniere.<sup>2</sup>

A later technique employed for the filling in of relatively small defects consisted in the removal of the adjacent outer table of a portion of the skull. Bone obtained in this manner was transferred to the defective area. More recently bone for large grafts has been obtained from the inner table of the ilium. However, because of the difficulty in obtaining sufficiently large bone grafts for extensive defects and of giving suitable curvature and shaping to the bone, interest in the utilization of nonirritating foreign materials was revived. The materials employed for this purpose are vitallium, tantalum, ticonium, and acrylic resins. Vitallium requires preliminary casting, a serious inconvenience. On the other hand, tantalum and ticonium can be applied in sheets that are easy to shape. These metals are difficult to obtain and expensive, however, aside from the disadvantage of always remaining foreign bodies. The tolerance of the tissues to acrylic resins has not as yet been fully ascertained.

In twelve cases recently operated on by Converse at the French Maxillo Facial and Plastic Surgery Center in Algiers consistently good results were noted in the repair of mandibular defects caused by war injuries. Chip-bone grafts removed from the ilium were used. As a result of this experience, the following technique has been evolved for the filling in of cranial defects with cast or formed masses of bone chips. This molding and casting technique has been used with excellent immediate results in three cases. Because of the extensive use made of molds and casts, their preparation is described in detail.

As a means of simplifying this text the following definitions are given. A *mold* is an impression of an original object. A *cast* is an impression from the

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mold. Accordingly, the mold is the *negative* and the cast is the *positive*. The cases described are repaired by incorporating with the bone surrounding the skull defect a cast area of bone chips that is of the desired thickness and shape to replace the lost contour and to give mechanical protection to the brain. In other words, a cast of bone chips is suitably shaped to restore the defective skull.

#### PREPARING THE MOLD

In preparing the metal mold for the bone chips the first step is to make an agar mold of the entire face of the patient. This procedure has been described by Clarke and Cone<sup>3</sup> and by Clarke.<sup>4</sup> From the agar mold is made a plaster of Paris cast as seen in Fig. 1. This too has been described in detail by Clarke.<sup>5</sup> It is of considerable advantage to make a cast of the entire face including the ears; the normal side then can be used as a guide for the restitution of the contour of the defective side.

In the case illustrated, the cerebrospinal fluid causes the skin over the defect to recede and expand. Restoration of the skull should aim at a replacement of the outer table to its proper level; also, it should aim at restoration of the normal curvature of the inner table to allow for expansion of the fluid. The plasteline is modeled into this area on the plaster cast and serves the purpose of fixing the size of the cavity, as well as affording support for the wax modeling which is to follow.

The amount of wax to be placed over this area will represent the thickness of the skull. This must be determined by considering sectional views of normal skulls.

It must be realized that this work is done over the skin surface as represented on the mold. For this reason it is necessary to consider the sectional view of the progressive work (Fig. 2). The top illustration shows the sectional view of the head with the wax and plasteline in place. The points *A* and *B* represent the area on which the bone graft eventually will rest on the natural bone of the skull. The skin flap will be turned back and the "bone plastic" put in place against the denuded surfaces of the bone of the skull. In this manner the skin shifts position to the outside of the bone plastic, as seen in the bottom part of Fig. 2, instead of lying on the inner side of the plasteline and wax reconstruction as seen in the top part of the same illustration.

After the plasteline has been put in place, as seen in the top part of Fig. 3, to represent the contour of the brain, a layer of modeling wax is applied to represent the thickness of the chip-bone graft. This wax is seen in the bottom half of Fig. 3. It is modeled so as to restore normal contours of the head. When both sides are symmetrical the modeling is completed.

A wax composition suitable for the purpose may be compounded from the following formula:

	Parts by weight
Paraffin	2
Resin (colophony)	2
Petroleum jelly	1

The paraffin and petroleum jelly are melted together. Then the resin is added and the mixture is heated slowly until the entire mass is melted. In



Fig. 1.—A and B, Case 3, Lt. B. Injury sustained from shell fragment. Skull defect 15 × 9 cm. C and D, The space to represent the inner table of the cranium is outlined on the plaster cast.



summer the petroleum jelly can be reduced or eliminated. In cold weather it may be necessary to increase the amount of petroleum jelly to have the material sufficiently plastic to work as a stiff clay between the fingers. This mixture becomes fairly hard on cooling.

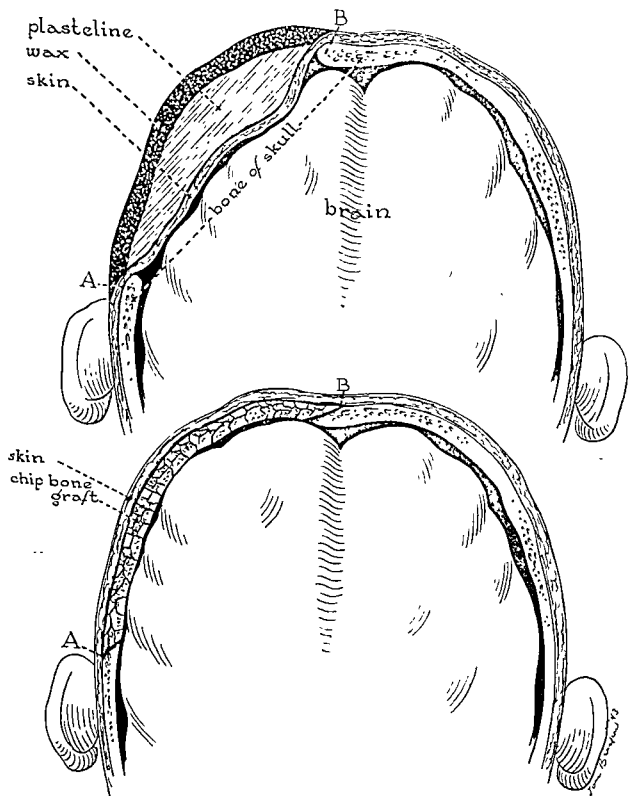


Fig. 2.—Sectional views of the head showing the wax and plasteline reconstruction and the final cast chip-bone graft. The points A and B represent the places where the chip-bone graft comes into contact with the normal bone.

When the modeling is completed and the wax mixture has cooled, a plaster mold is made over the modeled area as seen in Fig. 4A. This plaster mold is rubbed with talc, a separating medium, and then placed in French casting sand, Fig. 4B. The sand is patted flat around the mold to form an edge. The edge of the sand mold is dusted with talc to form a separation. A tin wall, Fig. 4C, is placed over the plaster mold. French casting sand is then put into



Fig. 3.—*A* and *B*, Plasteline is used to fill in the area that will represent the inner table of the cranium.  
*C* and *D*, The wax is used to reconstruct the contours of the head. This wax will also represent the shape and thickness of the cast bone graft.

the wall and packed against the plaster mold and the sides of the wall. It is finally packed tightly with a hammer as seen in Fig. 4D.

The *tin wall* containing the sand and plaster mold is then turned upside down and the plaster mold is removed as seen in Fig. 5A. The resulting half of the sand mold thus obtained is placed on a gas burner and baked hard and dry (Fig. 5B). It can then be poured with lead as shown in Fig. 5C. One-half of the metal mold that will be used for the bone chips is thus produced.

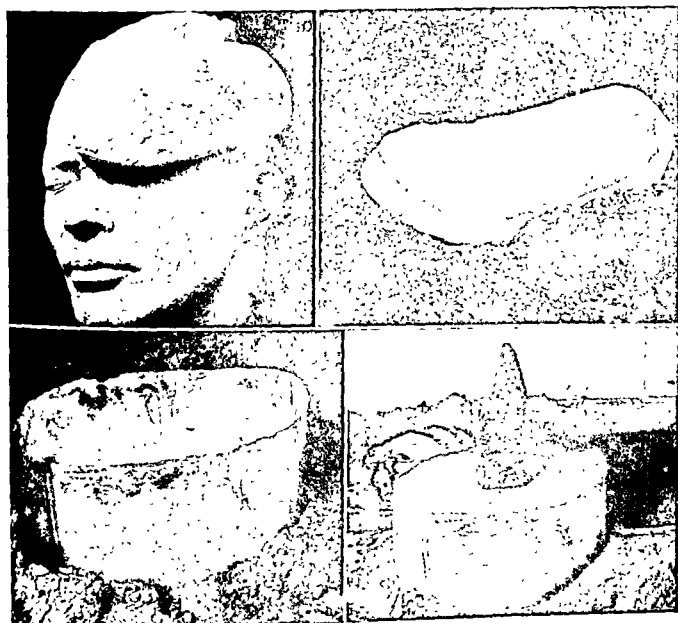


Fig. 4.—A, A plaster mold is made over the wax reconstruction.  
 B, The mold is removed and placed in French casting sand.  
 C, A tin wall is placed over the plaster mold.  
 D, The wall is filled with sand and tightly packed.

At this point it becomes necessary to remove the modeled wax piece from the plaster cast of the patient's head. If the head has been kept wet during the modeling, the wax will not stick to the plaster but will separate easily in one piece. This wax piece is imbedded in a two-piece plaster-silica mold as seen in Fig. 6. Such a mold can be made by mixing equal parts of plaster of Paris and either river sand or powdered silica while in a dry state. This is added to a predetermined amount of water as in the regular procedure of plaster casting and the mold is made. The resulting mold is opened and the wax pattern removed. The plaster-silica mold is closed and completely dried over a

gas burner. It is then tied together with wire, and melted lead is poured in through a funnel-like opening. When the lead has set and cooled, the plaster-silica mold is opened and the lead piece is removed. This will be a lead replica of the wax-modeled area and should fit the plaster cast of the patient's head in the same manner as the wax.

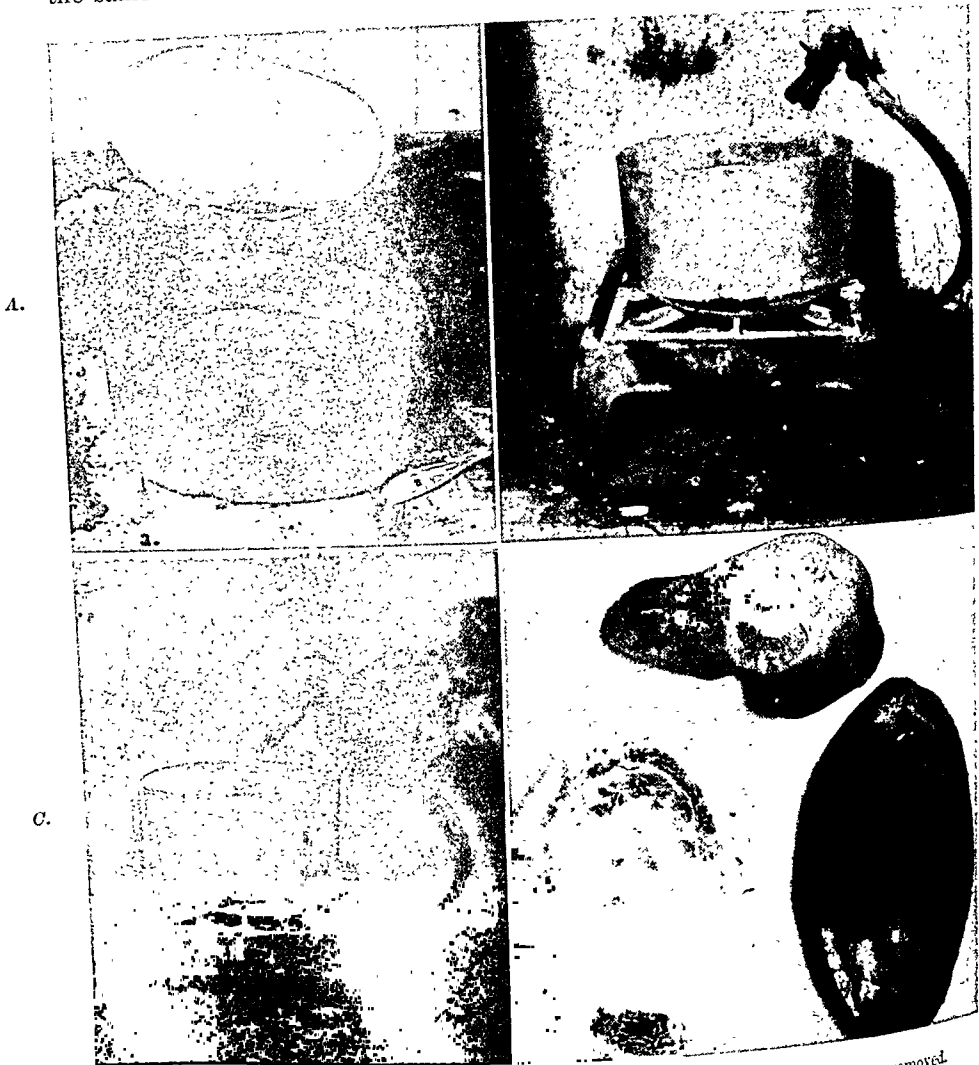


Fig. 5.—A, The sand mold is turned upside down and the plaster cast is removed.  
 B, The sand mold is baked hard and dry.  
 C, The sand mold is poured with metal.  
 D, Both pieces of the metal mold and the metal replica of the wax reconstruction.

This piece is now placed in the half of the lead mold which was prepared previously and should still be in the tin wall. A thin coating of plaster, water, and glue is painted over these lead pieces to facilitate separation of one metal part from another when the second half of the lead mold is poured. The plaster in the mixture serves to keep the plaster in this separating medium from setting and helps to hold it as a thin "coat of paint" to the set metal. The second half of the lead mold is then made. It is important to remember that the lead

must not be so hot as to melt the first half of the mold, however, it must be hot enough to flow freely. When the second half has been poured and has cooled, the mold is opened, thus producing two halves of the mold and the metal replica of the wax replacement as shown in Fig 5D. This is the mold which will be used to receive the bone chips. The metal replica of the wax modeling is of no further use and may be discarded. Fig. 7 is a mechanical drawing of a cross section of the metal mold or die and counterdie that is used to compress the bone chips.

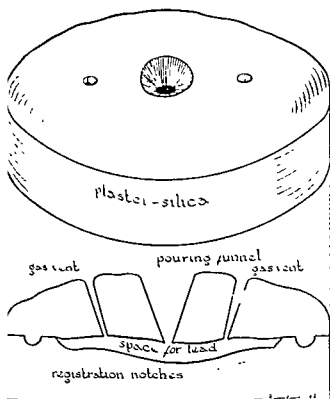


Fig. 6.

Fig. 6.—The plaster silica mold for making metal replica of wax reconstruction of chip-bone graft.

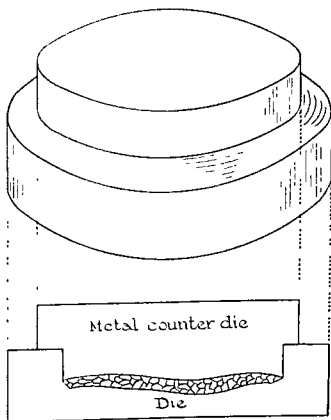


Fig 7.

Fig. 7.—The metal mold for making the cast chip-bone graft.

### THE SURGICAL PROCEDURE

The steps of the surgical procedure are: under general anesthesia (intratracheal, gas, oxygen, and ether), and after a peripheral infiltration of one per cent novocain and adrenalin, a wide scalp flap is outlined with pen, and ink and incised. This flap is carefully dissected from the dura, thus widely exposing the defect (Fig. 8). The edges of the bone defect are resected in a beveled manner and the defect is then ready to receive the bone graft.

The inner table of the ilium is exposed by elevating the periosteum from the crest (top illustration in Fig. 9). A segment of the inner table is removed and sectioned into cube fragments measuring 0.25 to 1 cm. These bone fragments are packed into the lower half of the sterilized metal mold (Fig. 9B). The bone fragments are added to the mold to a height above that of the desired thickness of the final bone graft to insure impaction of the fragments after they have been compressed. Blood from the iliac wound is then withdrawn in a syringe and mixed with the bone fragments in the mold. The upper half

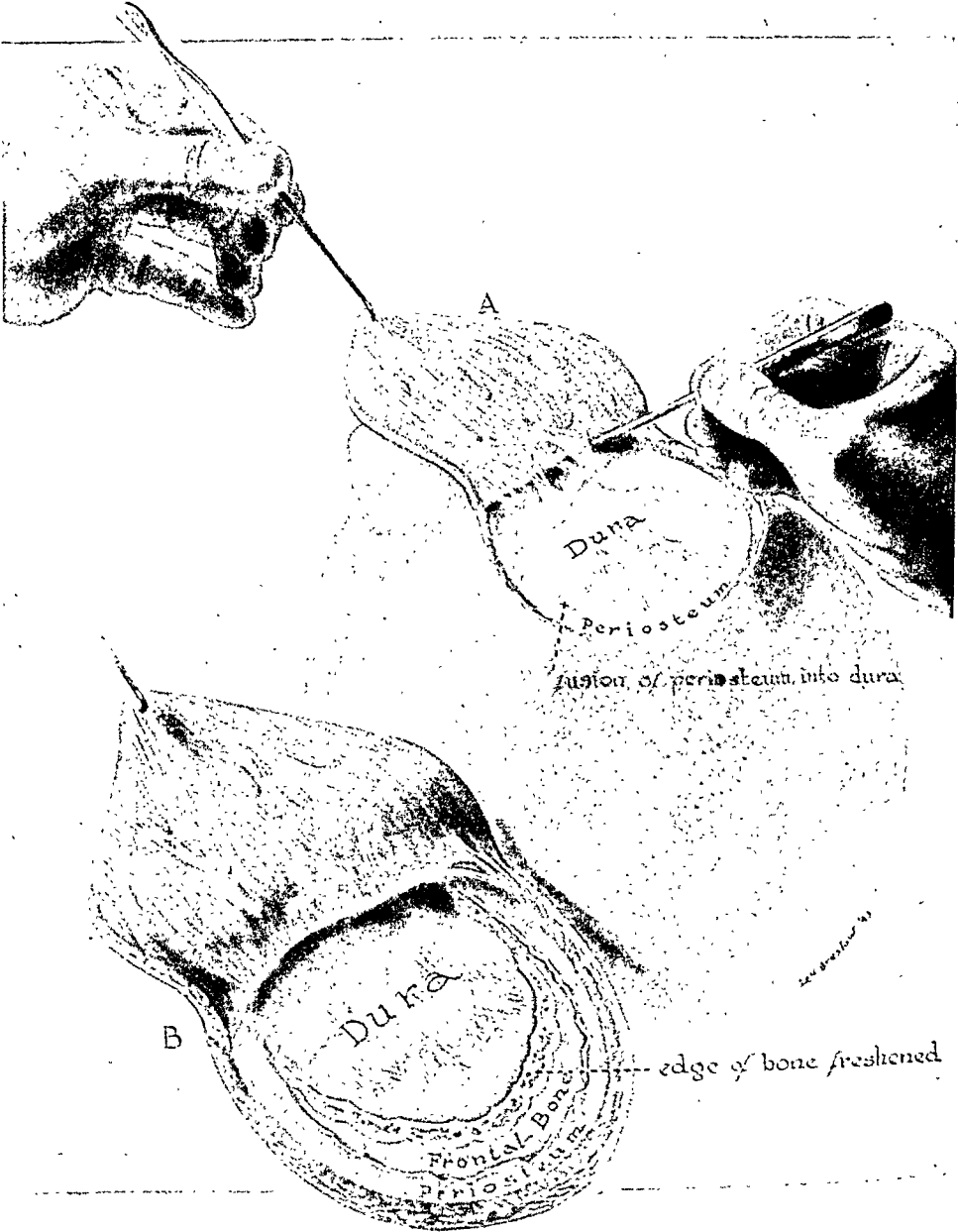


Fig. 8.—1, This illustration shows an extensive defect over the frontal lobe. The skin flap has been dissected away from the dura.  
B, The bone edges have been beveled and made ready to receive the graft.

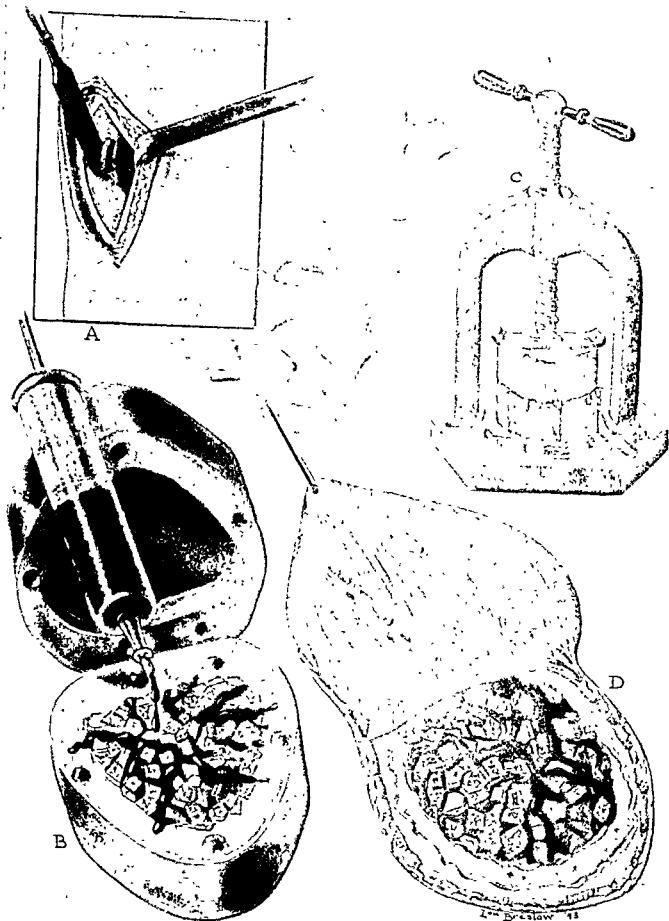


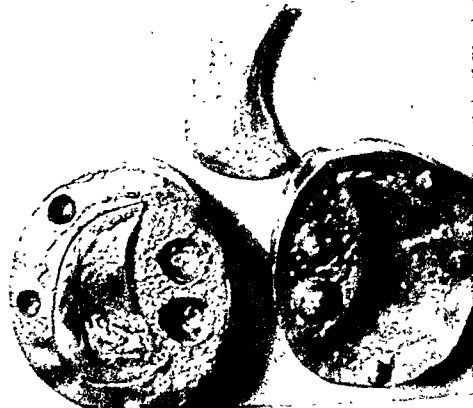
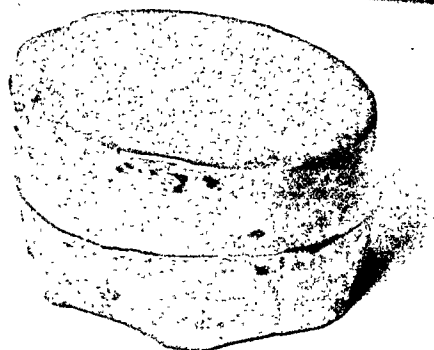
Fig. 9.—A, This illustration shows the removal of bone chips from the inner table of the skull.  
 B, The bone chips are packed into the mold. Blood is poured over the chips.  
 C, The upper mold is put into position and the cast bone graft is made in a dental or letter press by the joint action of compression and coagulation of the blood.  
 D, The cast bone graft is put in place and the flap is ready to be reapplied.



Fig. 10.—Case 1, Sgt. G. An injury sustained in the bombing of a train. The anterior and posterior walls of the left frontal sinus were destroyed, leaving an opening  $6 \times 3.5$  cm.



B.



D.

Fig. 11.—A, Case 1. A plaster cast of the patient's left frontal region showing the defect.  
 B, A plaster cast showing the metal reconstruction.  
 C, The closed metal mold for compressing the bone chips.  
 D, The open metal mold with the metal cast. This replica was used only in making the mold and was finally discarded.



of the mold is put in place and the entire mold is inserted into a sterilized strong dental or letter press (Fig. 9C). Pressure is applied and maintained until the blood has clotted, the fibrin of the clot acting as a cement in holding together the bone particles under pressure. This bone "plastic," shaped, cast, and held in one mass by the double action of compression and fibrin cement, is then introduced into the position covering the defect of the skull (Fig. 9D).

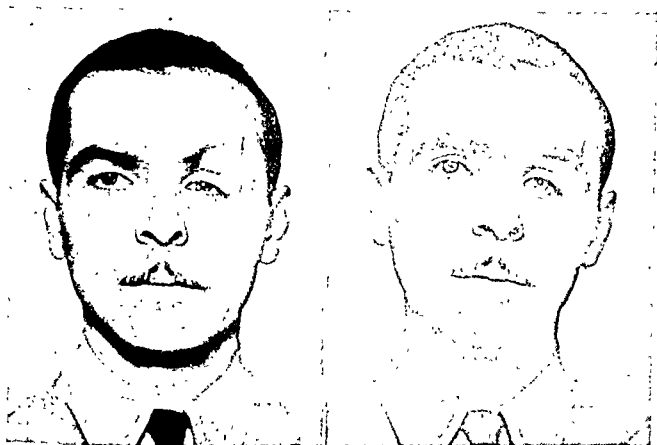


Fig. 12.—Case 1. Left: The restoration of the contour by the use of a chip-bone graft. Right: A cosmetic pencil was used to reconstruct the eyebrow. The ptosis of the upper left eyelid resulted from a partial destruction of the levator and superior oblique muscles.

It is important to note that the fragments must be sufficiently pressed together so that the cast chip-bone graft will emerge from the mold as one solid mass. Failure to do this results not only in irregularities on the surface of the graft but also in a flattening of the curvature of the graft, which would prove detrimental to the contour restoration.

Immediate follow-up studies on the three cases reveal the following facts:

1. Cases 1 and 2 presented a resistant, firm, and shapely contour restoration after five and four months respectively. In Case 2 a few small irregularities that were present have become smooth. Roentgenographs taken after the operation suggest that the numerous fragments have become knitted into a uniform mass.

2. Case 3 represented a large defect which was filled by the cast bone graft. However, because of the fact that the mass was not sufficiently compressed, some flattening took place. This was easily corrected in a second surgical operation. In such a large defect it should also be possible to incorporate in the bone mass one or two tantalum-strip supports or a tantalum wire mesh if necessary.



Fig. 13.—A and B, Case 3, Lt. B, before the operation.  
C and D, The same patient 27 days after the operation.

## CONCLUSIONS

The method described was used in three cases. The first operation of this type was performed in August, 1943. It is still too early (December, 1943) to predict whether the grafts now in place will give ultimate clinical satisfaction. The final clinical results obtained in these cases will be reported later.

The technique represents a considerable amount of work in preparing the casts and molds before the operative procedure, but the surgery is thus simplified. The shaping of the graft is done by the mold, and the contour, although it must be predetermined, may be excellent.

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Erratum

In the article "A Solid Medium for the Transportation of Delayed Gonococcus Cultures" by Nell Hirschberg in the March issue of this JOURNAL 29: 314, 1944, page 315, line 37:

0.24 per cent Nile blue A should read 0.024 per cent Nile blue A.



Fig. 13.—A and B, Case 3, Lt. B, before the operation.  
C and D, The same patient 27 days after the operation.

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## *CLINICAL AND EXPERIMENTAL*

### THE ACUTE AND CHRONIC TOXICITY OF ISOPROPYL ALCOHOL<sup>\*</sup>

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THE similarity in physical properties of isopropyl and ethyl alcohol has suggested the substitution of the former for the latter. Although not a complete substitute in every instance, isopropyl alcohol has found considerable use in industry. Industrial toxicology of the alcohol as the result of occupational exposure apparently has not been a serious problem. However, with the present acute shortage of ethyl alcohol, a wider application of isopropyl alcohol is in prospect, especially for cosmetic and hygienic purposes. For this reason its acute and chronic effects assume a greater importance than heretofore.

Isopropyl alcohol has been of considerable scientific interest in the past, and several summaries of the literature have appeared, notably those of Munch and Schwartz<sup>1</sup> and Morris and Lightbody.<sup>2</sup> Most of the published reports have been confined to acute effects as observed in various animals, special organs, and microorganisms, using a variety of methods. In general, these investigations agree that isopropyl alcohol is approximately twice as active in its toxic propensities as ethyl alcohol.

Studies on chronic intoxication have not been very extensive. Pohl<sup>3</sup> administered orally a total of 224 c.c. of isopropyl alcohol in small daily doses during a period of two months to a puppy and found that the alcoholized animal showed a greater gain in weight than an untreated litter mate. Boruttau<sup>4</sup> in a similar experiment gave 5 c.c. per kilogram for several weeks to one dog and observed no harmful effects. Morris and Lightbody<sup>2</sup> reported experiments

<sup>\*</sup>From the Department of Pharmacology and Therapeutics of the College of Medicine, Wayne University. This report is part of the project which involves a complete pharmacologic investigation of isopropyl alcohol and is supported by the Standard Alcohol Company of New York, through courtesy of Mr. James Park.

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on rabbits, the object of which was to determine the development of tolerance. They found a decreased rate of recovery from the narcotic effects during the last four days as compared with the first four days of an eleven-day observation period. This was ascribed by them to an accumulation of alcohol, because of slow destruction and elimination, and to the production of a depressant intermediary metabolite, probably acetone.

Much of the earlier data are subject to considerable variation, probably the result of using an alcohol of doubtful purity<sup>3</sup> as well as the employment of different doses and methods of administration. In view of this fact and also in anticipation of projected experiments on absorption, combustion, and distribution of isopropyl alcohol, it seemed necessary to re-establish minimum lethal doses with a product of known purity and with adherence to a standard technique. Therefore, we undertook a comprehensive study of acute and chronic toxicity of isopropyl alcohol, employing a product at least 99 per cent pure. Some comparisons with ethyl alcohol have also been made.

#### HEMOLYTIC ACTION

Based on the evidence<sup>2</sup> that isopropyl alcohol is slowly destroyed and eliminated, suggesting a prolonged sojourn in the blood stream, hemolysis of blood cells and precipitation of blood proteins may occur. This continued injury, should it develop, could be responsible for the reported delayed deaths in animals. The importance of the blood effects also becomes apparent in metabolic studies where prompt and complete absorption of any specific dose must be assured and intravenous administration is the method of choice. Hemolytic actions were observed *in vitro*, using a technique similar to that employed in testing red cell fragility. One cubic centimeter of whole rabbit blood was added to a series of tubes containing isopropyl alcohol in concentrations ranging from 0 to 100 per cent in 10 per cent increments, with and without sodium chloride. All tubes were inspected at the end of three and twenty-four hours. Isopropyl alcohol in water produced immediate hemolysis in 10 and 20 per cent concentrations. Dilutions prepared with 0.9 per cent sodium chloride offered complete protection in the 10 per cent tube, but produced complete hemolysis in the 20 per cent tube at the end of three hours. This result was not significantly altered when final concentrations of sodium chloride in each tube were maintained at 0.9 per cent. Concentrations of isopropyl alcohol above 20 per cent regardless of the diluent produced precipitation of blood proteins, the amounts of precipitate varying directly as the alcoholic concentrations increased. It appears that exposure of blood to 10 per cent isopropyl alcohol in 0.9 per cent sodium chloride is a condition which if it obtained within the circulatory system, would not produce any grossly detectable injury to this tissue. This is at least twenty times the blood alcohol concentrations which could be reached with even a fatal dose, and delayed effects of such exposures would not seem to be a contributory cause of death.

#### INTRAVENOUS TOXICITY

This was studied in ten rabbits and seven dogs which received isopropyl alcohol by slow infusion into a suitable vein, a 20 per cent solution in 0.9 per cent sodium chloride being given at the rate of 0.25 c.c. per kilogram per min-

ute. This slow infusion rate could not be maintained in rats, and after ten trials with widely varying results no further attempts were made. The anesthetic dose was also determined as the amount necessary to produce elimination of the corneal reflex. Table I shows the average anesthetic and fatal doses for the alcohol in rabbits and dogs.

TABLE I

AVERAGE ANESTHETIC AND FATAL DOSES OF ISOPROPYL ALCOHOL ACCORDING TO INTRAVENOUS INFUSION IN RABBITS AND DOGS

ANIMAL	NUMBER OF EXPERIMENTS	ANESTHETIC DOSE C.C. PER KILOGRAM	FATAL DOSE C.C. PER KILOGRAM
Rabbit	10	1.25	8.23
Dog	7	1.15*	5.12

\*An average for five dogs.

It is seen that the anesthetic dose for both species is almost identical, but represents only 39 per cent of the fatal dose for rabbits as against 65 per cent of the fatal dose for dogs.

#### GASTRIC TOXICITY

Isopropyl alcohol was administered gastrically to one hundred and forty white rats, thirty-five rabbits, and one hundred and two dogs. Food was withdrawn from all animals for twenty-four hours before administration. Suitable sounds or stomach tubes were employed, the alcohol being introduced as a 50 per cent solution in 0.9 per cent sodium chloride or water in rats and rabbits. Dogs did not tolerate the alcohol well. After some preliminary trials it was found that a 25 per cent concentration in warm tap water was the most suitable. Vomiting occurred in sixty-two dogs and these animals were discarded. A period of three days was allowed for a fatal outcome. The results are shown in Table II.

TABLE II

TOXICITY OF ISOPROPYL ALCOHOL ACCORDING TO GASTRIC ADMINISTRATION IN RATS, RABBITS, AND DOGS

DOSE C.C. PER KG.	WHITE RATS		RABBITS		DOGS*	
	NUMBER OF ANIMALS USED	PER CENT MORTALITY	NUMBER OF ANIMALS USED	PER CENT MORTALITY	NUMBER OF ANIMALS USED	PER CENT MORTALITY
8.0	10	100			1	100
7.5	20	75	5	100	3 (2)	100
7.0	25	44	5	100	6 (4)	67
6.5	25	56	10	90	9 (5)	55
6.33			10	0		
6.17			5	0		
6.0	20	25			8 (9)	37
5.5	15	13			5 (23)	60
5.0	15	7			6 (8)	33
4.5					2 (11)	0
2.5	10	0				

\*Emesis was commonly exhibited in dogs, and the number discarded therefore on each dose appears in parentheses.

The surely fatal dose in white rats was 8.0 c.c. per kilogram; in rabbits 7.0 c.c. per kilogram; and in dogs 7.5 c.c. per kilogram. The 50 per cent killing dose for each species, as calculated by the method of Reed and Muench,<sup>6</sup> was 6.73 c.c., 6.41 c.c., and 6.15 c.c. per kilogram or 84 per cent, 91 per cent, and 82 per cent of the surely fatal dose for rats, rabbits, and dogs, respectively.

## CHRONIC TOXICITY

The cumulative effects of isopropyl alcohol in animals might be reflected in growth and body-weight changes after imbibition of the alcohol for prolonged periods. Intermediate oxidation products or the alcohol as such could be responsible for tissue damage. For a study of the effects of chronic intoxication the procedure of voluntary drinking of isopropyl alcohol by white rats was employed.

Seven groups of five rats each of about 50 grams body weight were placed in cages and given free access to Steenbach's whole artificial diet.\* Two groups, one of males and another of females, were given water to drink and thus served as controls. The other groups were given 0.5 (males), 1.0 (females), 2.5 (males), 5.0 (females), and 10.0 (males) per cent solutions of isopropyl alcohol in water. The rats were weighed weekly, the food weighed as often as necessary, and fluid intake was recorded daily. This regimen was maintained for twenty-seven weeks, and when the experiment was terminated, all survivors were sacrificed with the exception of three females from each of the control, 1.0 and 5 per cent groups; the latter were used to study the effects of isopropyl alcohol on reproductive functions. Tissues from the sacrificed animals were submitted to Dr. Mark E. Maun of the Department of Pathology, for pathologic study. The essential experimental data are presented as curves in Fig. 1, and show daily changes in body weight, daily food consumption, daily intake of fluid, and daily dose of isopropyl alcohol for all rats from week to week except those drinking 10 per cent alcohol.

The results were as follows: the rats which received 10 per cent isopropyl alcohol refused to drink this fluid and died in from seven to twenty-eight days; hence the results were discarded. Female rats drinking 1 per cent and 5 per cent alcohol remained well throughout the observation period. In the 0.5 per cent group one animal died during the fifth week, and a second rat succumbed the sixth week. In the 2.5 per cent group, deaths occurred in the eighth, twentieth, and twenty-second week. Whether these deaths were due to natural causes or to a greater susceptibility of the male rat to isopropyl alcohol could not be ascertained, as the rats were found dead unexpectedly, and extensive post-mortem changes precluded an accurate diagnosis by the pathologist.

Growth and body weight curves of the surviving males in each group showed some retardation during the first thirteen weeks of observation, or about 5 per cent and 17 per cent, respectively. A definite gain was noted in the last fourteen weeks. Actually weights were 5.6 per cent greater for the 0.5 per cent group and 9.1 per cent greater for the 2.5 per cent group than those of the controls at the end of twenty-seven weeks. Since it is known that isopropyl alcohol is burned in the body, the increased caloric intake may, in part at least, account for these increases. Female rats on 1 per cent and 5 per cent alcohol showed some depression in growth and body weight, which at the termination of the experiment amounted to a 12 per cent loss in the former group and a 10.3 per cent loss in the latter group. Food intake in all rats showed that appetite was not impaired, the quantity consumed by the test animals being equal to or

\*This diet contained: feed corn meal, 68 parts; linseed oil cake meal, 10 parts; dried ground alfalfa, 2 parts; powdered casein, 10 parts; Crisco, 5 parts; cod liver oil, 3 parts; bone ash, 1.5 parts; and sodium chloride, 0.5 part.



greater than that of the control animals. There was a tendency for a decreased fluid intake as alcoholic concentrations increased, which is to be attributed partially to the unpalatability and partially to the depressant action of the alcohol.

The amounts of isopropyl alcohol ingested were quite large, the daily intake averaging about  $\frac{1}{11}$ ,  $\frac{1}{6}$ ,  $\frac{3}{8}$ , and  $\frac{5}{8}$  of the oral, surely fatal, dose in ascending order of concentration. Careful notes were made on the behavior of the alcoholized rats, but aside from the slight retardation of growth and probable accidental death of some rats in the 0.5 per cent and 2.5 per cent groups, no

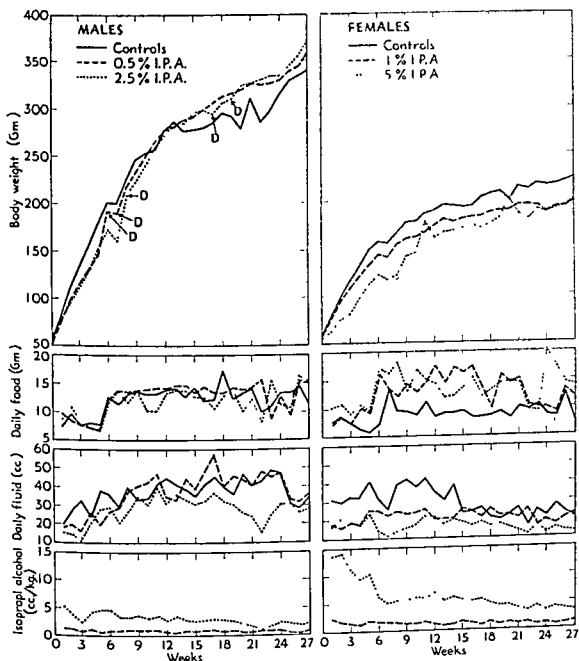


Fig. 1.—Effect of continued drinking of isopropyl alcohol upon white rats. *D* represents the death of one animal at the time. I.P.A. = Isopropyl alcohol.

definite manifestations of poisoning were observed. Gross and microscopic examinations of the brain, pituitary, lung, heart, liver, spleen, kidneys, and adrenals showed no evidence of gross or pathologic changes. It would seem, therefore, that retardation of growth and body weight loss were general physiologic effects rather than reflections of injury to some specific function or organ.

#### COMPARATIVE TOXICITY

Correlation of the toxicity of isopropyl and ethyl alcohols was established in rats, rabbits, and dogs. These were limited to the oral route since there is no

known indication for the use of isopropyl alcohol intravenously, and the more important aspects are associated with gastric ingestion. The 50 per cent fatal dose was established for ethyl alcohol in fifty rats, thirty-five dogs, and sixteen rabbits; however, because of the similarity of the results of our experiments with those of Morris and Lightbody,<sup>2</sup> the more expansive data of the latter investigators were utilized to calculate the M.L.D. 50 dose in rabbits. A summary is presented in Table III.

TABLE III

COMPARATIVE TOXICITY OF ISOPROPYL AND ETHYL ALCOHOL ACCORDING TO ORAL ADMINISTRATION IN RATS, RABBITS, AND DOGS

ANIMAL	M.L.D. 50 IN C.C. PER KILOGRAM		RATIO ETHYL = 1.0
	ISOPROPYL	ETHYL	
Rat	6.73	12.96	1.92
Rabbit	6.41	10.22	1.56
Dog	6.15	12.25	1.99

The results are in agreement with those obtained by other investigators and indicate that isopropyl alcohol is approximately twice as toxic as ethyl alcohol. If this relationship holds for man, the surely fatal dose of isopropyl alcohol may be calculated from the data supplied by Jetter.<sup>7</sup> He reported two confirmed deaths from acute alcoholism. Blood alcohol concentrations were 480 mg. per 100 c.c. and 470 mg. per 100 c.c., and, according to Newman,<sup>8</sup> this represented the rapid imbibition of about one and a half pints of whiskey. It follows that approximately 166 c.c. of isopropyl alcohol could be a fatal dose for man. When the emetic action of the alcohol is taken into consideration, it is extremely doubtful whether this quantity would be tolerated as a single dose. Furthermore, depression of the central nervous system, which appears with a dose considerably below the fatal dose as observed in animals, would limit ingestion, and the combination of the emetic and depressant actions would definitely lessen the probability of ingesting a fatal quantity.

## COMMENT

Throughout the course of our experimental work the general effects of isopropyl alcohol followed closely the sequence of toxic actions of ethyl alcohol. The actions of significant doses upon the central nervous system were depression and later paralysis. Although nystagmus was repeatedly observed, other ocular symptoms such as impairment of vision as determined subjectively were not seen in the several hundred animals used. Salivation, retching, and vomiting were somewhat less frequent in the ethyl alcohol series of dogs than in the isopropyl alcohol series; actually, an incidence of 48.3 per cent for the former and 60.7 per cent for the latter. Vomiting in the isopropyl alcohol series occurred at two stages of intoxication; early, or within five minutes after administration of the alcohol and which was undoubtedly due to distention and irritation, and later, after absorption had progressed to the coma state but with the corneal reflex still present. This delayed effect was attributed to a central action, since vomiting was observed in two dogs which received intravenous toxic doses, and retching was a rather frequent occurrence. These observations do not differ from those of ethyl alcohol as reported by Newman in his review already referred to.

Delayed toxic effects as suggested by Morris and Lightbody have not been observed. All of the fatalities among the animals to which the alcohol was administered orally occurred within twenty-four to thirty-six hours, and if any animal showed signs of recovery before this time interval, complete return to normal insofar as appetite and behavior were concerned was assured. To test the matter further, two dogs were given one-half, and three dogs three-fourths, of the intravenous fatal dose and allowed to recover. Within twenty-four hours no symptoms of intoxication were apparent and the animals remained well. The rather slight effects on growth and body weight in rats in chronic intoxication would also seem to rule against any delayed toxic action.

#### CONCLUSIONS

1. Isopropyl alcohol, as determined in several species of animals after various routes of administration, is approximately twice as lethal as ethyl alcohol.
2. The continuous consumption of isopropyl alcohol in doses of 0.75 to 5.28 c.c. per kilogram per day may either augment growth and body weight in rats or produce a slight decrease.
3. Evidence of delayed toxic effects was not observed, and the results of chronic intoxication would seem to argue against the production of a harmful intermediary metabolite.
4. The effects of isopropyl alcohol in animals are similar to those of ethyl alcohol. Salivation, retching, and vomiting appear more frequently, but the general sequence of terminal symptoms proceeding to paralysis is similar to that elicited by ethyl alcohol.

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# THE TREATMENT OF PNEUMOCOCCAL PNEUMONIA WITH SULFAMETHAZINE\*

## PRELIMINARY REPORT

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SULFAMETHAZINE, 2-(4'-aminobenzene-sulfonylamino 4:6-dimethylpyrimidine), was first used in the treatment of pneumonia by Macartney et al., in Manchester, England.<sup>1</sup> Subsequently, Jennings,<sup>2</sup> also in England, reported the treatment of pneumonia with this drug in infants and children. These workers found that sulfamethazine, as well as being therapeutically effective, caused very few toxic reactions.

Rose, Martin, and Bevan<sup>3</sup> reported the results of studies on the chemical, pharmacologic, and therapeutic properties of sulfamethazine. Comparing sulfamethazine with sulfapyridine, they found that sulfamethazine, administered over a period of two weeks to albino rats, did not produce renal damage, while sulfapyridine, similarly administered, produced pathologic changes in the kidneys like those caused by sulfathiazole. In human beings, despite the fact that high blood concentrations were obtained clinically, crystalluria and hematuria never occurred. This was attributed to the fact that both sulfamethazine and acetyl sulfamethazine were highly soluble in Ph range of 5.5 to 8 (normal Ph range of urine).

We reported recently<sup>4</sup> the results of a comparative study on the treatment of Type I pneumococcal infections in albino rats in which sulfathiazole, sulfadiazine, and sulfamethazine were used alone or in combination with Type I antipneumococcal serum. We found that sulfamethazine alone, and when combined with serum, produced respectively higher survival rates than any of the other sulfonamides similarly employed.

It is the purpose of this paper to present and analyze the results of treatment with sulfamethazine in forty-eight cases of pneumococcal pneumonia.

*Plan of Study.*—During the three months period, from March 1 to May 31, 1943, forty-eight adult patients (over 15 years of age), with pneumococcal pneumonia, were admitted to the medical services of the Long Island College and Kings County Hospitals in Brooklyn. In each of these cases, isolation of pneumococci from the sputum was accomplished, and together with a typical

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The sulfamethazine used in this study was donated by the Nepera Chemical Company.

symptomatology, physical and roentgenographic findings established the diagnosis of pneumococcal pneumonia. Treatment, however, in no case was delayed until the causative organism was identified, but was begun as soon as the existence of pneumonia had been clinically determined.

The pneumococci were identified by direct Neufeld typing which was confirmed by the typing of the peritoneal exudate from mice inoculated with the sputum. Cultures of the blood were made upon admission to the hospital and were repeated daily during the febrile period. Blood studies, including red and white blood cell counts, hemoglobin, and urea nitrogen determinations were made at least every other day. Daily examinations of the urine were made for the presence of crystals of sulfamethazine and acetyl sulfamethazine. Determinations of both free and total concentrations of sulfamethazine in the circulating blood were made at daily intervals.

AVERAGE FREE AND TOTAL BLOOD SULFAMETHAZINE LEVELS AND  
AVERAGE PERCENTAGE OF CONJUGATION IN  
PNEUMOCOCCAL PNEUMONIA

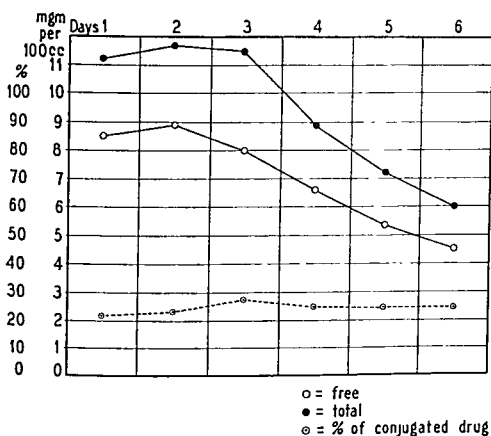


Chart 1.

*Dosage of Sulfamethazine.*—All the patients were given an initial dose of four grams orally followed by one gram every four hours day and night until the temperature was normal for approximately sixty hours. With this dosage, adequate blood levels were usually obtained. On the first two days of treatment, free sulfamethazine in the blood averaged 8.76 mg. per 100 c.c. with extremes of 1.3 and 16.0 mg. per 100 c.c. The average degree of conjugation during the first six-day period, on the last day of which 29 per cent of the patients were

represented, averaged 24.0 per cent, a peak of 25.7 per cent having been reached on the fourth day (Chart I), with extremes of 0 per cent and 55 per cent (Chart II).

*Analysis of Cases* (Table I).—Nineteen types of pneumococci were found in the sputum or sputum and blood of these forty-eight cases. Type III pneumonia, occurring in six cases, was the most frequently encountered. There were four cases each of Types II, VII, VIII, and "x," and three each of I, V, and XVIII. Bacteremia occurred in 10, or 20.8 per cent of the cases, there being two each of Types I and II, and one each of Types III, IV, V, IX, XII, and XIV.

Antecedent diseases of significant degrees of severity were encountered in twenty-two cases (Table I). The ages of thirty-eight patients ranged between the fourth and seventh decades inclusively (Table II). The duration of illness and lobar distribution of the pneumonia are shown in Tables III and IV.

#### FREE AND TOTAL BLOOD SULFAMETHAZINE LEVELS IN 48 CASES OF PNEUMOCOCCAL PNEUMONIA

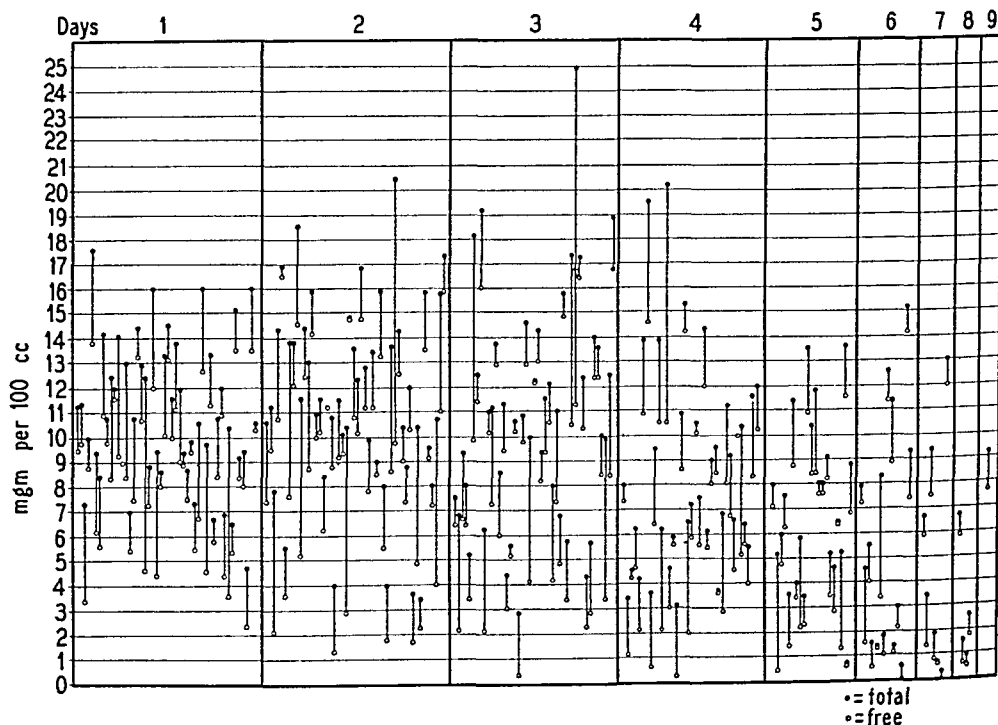


Chart 2.

*Results of Treatment* (Table I).—All of the patients, except three in the sixth, seventh, and eighth decades respectively, recovered. Each of these three fatal cases had bacteremia and severe antecedent disease conditions to which apparently the pneumonia was a secondary and terminal condition. One, a 75-year-old white man, with Type I pneumonic involvement of the right middle lobe, had generalized arteriosclerosis and uremia and had been ill for six days before institution of therapy. Another, a 59-year-old white man, with chronic

alcoholism and delirium tremens, and Type XII pneumonia of the right middle lobe, had been ill for two weeks prior to admission. The third, a 65-year-old white man, with Type XIV pneumonia of the right lower lobe, had been ill for two weeks before admission, and had, in addition, arteriosclerotic and hyper-

TABLE I  
ANALYSIS OF CASES

TYPE	NO.	BAC- TEREMIA	ANTECEDENT DISEASES	COMPLICATIONS	TOXIC REACTIONS	DEATHS
I	3	2	Rheum. H. D.		Drug Fever(1)	
II	4	2	Tuberculosis Senility, Malnutrit. Arteriosclerosis Uremia			1 <sup>(a)</sup>
III	6	1	Chr. Leucemia Tuberculosis Diabetes Mellitus	St. Effusion	Drug Fever(1)	
IV	2	1				
V	3	1	Tuberculosis			
VI	2		Bronchiectasis			
VII	4		Rheum. H. D.(2)	St. Effusion		
VIII	4		Art. Sci. H. D. Carcinoma Bronchiectasis Syphilis	St. Effusion(2)		
IX	1	1		St. Effusion		
X	2		Tuberculosis Art. Sci. H. D.			
XI	1		Tuberculosis			1 <sup>(b)</sup>
XII	2	1	Chr. Alc; D. T. Art. Sci. H. D. Uremia			1 <sup>(b)</sup>
XIV	1	1	Art. Sci., Hyper. H. D. Cong. Failure Pulmonary Edema Hypertension	St. Effusion		
XVIII	3					
XIX	1					
XXIV	2		Chr. Alc; Pulm. Tbc. Diabetes Mellitus			
XXXII	1					
XXXIII	2					
"X"	4					
Total	48	10			2	3
Per cent		20.8			4.2 per cent	

Fatality Rate: Gross 6.3 per cent.

Corrected 0 per cent.

Fatality Rate: Nonbacteremic 0 per cent.

Fatality Rate: Bacteremic: Gross 30 per cent.

Corrected 0 per cent.

TABLE II  
AGE DISTRIBUTION OF PATIENTS

AGE (YEARS)	NO. OF PTS.	DEATHS
10-19	2	
20-29	5	
30-39	12	
40-49	13	
50-59	5	1
60-69	8	1
70-79	3	1
	48	3

tensive heart disease and congestive failure; death was attributed to acute pulmonary edema.

The gross fatality rate was 6.5 per cent; the nonbacteremic fatality rate, 0 per cent; the gross bacteremic fatality rate, 30 per cent. The gross fatality rate can be corrected to 0 per cent if the above three cases in which the pneumonia was a terminal affair are excluded.

The clinical response was usually fairly rapid. The temperature fell to, and remained normal in an average of 55 hours, when three patients having febrile periods of 120, 136, and 216 hours (the last, a case of pulmonary tuberculosis) were excluded.

TABLE III  
DURATION OF ILLNESS BEFORE INSTITUTION OF THERAPY

NO. OF DAYS	NO. OF PATIENTS	DEATHS
1	8	
2	4	
3	4	
4	1	
5	0	
6	4	1
7	7	1
8 plus	12	1
Undetermined	8	
	48	3

TABLE IV  
LOBAR DISTRIBUTION OF PNEUMONIC INVOLVEMENT

LOBES	NUMBER OF PATIENTS	DEATHS
Right upper	3	
Right middle	2	2
Right lower	19	1
Left lower	17	
Left upper	0	
Multilobar		
Right	2	
Left	1	
Bilateral	4	
Total	48	3

The blood cultures in those patients with bacteremia were rendered sterile within twenty-four hours after beginning sulfamethazine therapy.

There were no purulent pneumococcal complications, and the only complications encountered were six instances of sterile pleural effusion.

*Drug Toxicity.*—The only toxic reactions observed were two instances of drug fever. Nausea and vomiting did not occur. Crystalluria and hematuria were never encountered, and none of the patients developed any increase in blood urea nitrogen. In two cases, the white blood cells dropped temporarily on the second day of treatment from 8,700 and 5,000 to 3,900 and 2,100 per cm. respectively at the time of crisis. There were no dermatologic nor neurologic reactions.

#### SUMMARY

The results of treatment with sulfamethazine in forty-eight cases of pneumococcal pneumonia are presented.



The gross fatality rate in this group of cases, of which 20.8 per cent had bacteremia, was 6.2 per cent; the corrected fatality rate was 0 per cent.

The only toxic effects noted were two instances of drug fever. There were no instances of crystalluria nor hematuria despite the fact that there were high concentrations of sulfamethazine in the blood.

The members of the staffs of the hospitals named, notably Doctors Tasker Howard, George H. Roberts, Henry Wolfer, and Joseph G. Terrance, permitted us to treat patients on their services with sulfamethazine.

We should like to express our appreciation to Doctors Janet Watson, Henry Chin, Benjamin C. Berliner, Julius Wolfe, Herbert Bermont, and Jacob Halpern for their clinical assistance, and to Miss Helen Sage for her technical assistance. In addition, we wish to thank Sidney M. Karlton for his cooperation throughout this study.

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THE PATHOLOGIC CHANGES PRODUCED BY PROLONGED ADMINISTRATION OF SULFAPYRAZINE AND SULFAMETHYLDIAZINE (SULFAMERAZINE) IN THE KIDNEYS OF RABBITS AS COMPARED WITH SULFATHIAZOLE AND SULFADIAZINE\*

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LORAINÉ GROSKIN LINTON, B.Sc.

THE occurrence of severe damage of the kidneys after prolonged administration of sulfonamides makes it necessary to subject this unwelcome symptom to special investigation. In many cases clinical observation and autopsies have revealed the pathogenesis and the site of injury in the urinary organs observed after administration of the various sulfonamide compounds. In addition, animal experimentation has given ample opportunity for comparing, at a given dosage schedule, the nephrotoxic effect of the compounds and for studying the pathologic tissue changes produced by prolonged drug administration.

A great many of the sulfonamide derivatives now in common use and several new products have been tested in this respect.<sup>1-10</sup> Judging from the literature, however, there exist differences of opinion concerning the following questions: (1) which derivative offers the highest chemotherapeutic activity associated with the highest possible security against kidney injury; (2) which doses and which period of daily administration are sufficient with the various derivatives to produce pathologic changes in the urinary organs; (3) which derivative has shown the greatest ability for the formation of gross concretions (uroliths) in the urinary passages?

In particular, the occasional observation of renal complications associated with the clinical use of sulfathiazole and sulfadiazine, such as hematuria, uroliths, suppression of the urinary output, stimulated the research for new derivatives of an equal therapeutic but a less nephrotoxic activity. For this reason especially, the para-isomer of sulfadiazine (sulfapyrazine) and both the dimethyl derivative of sulfadiazine (sulfamethazine, sulfamethylthiazole) and the monomethyl derivative (sulfamerazine, sulfamethyldiazine) were submitted to experimental investigation.

In our experimental study we were concerned with determination of the extent and site of pathologic changes produced in the kidneys of rabbits by prolonged oral administration of sulfapyrazine and sulfamethyldiazine (sulfamerazine) as compared with sulfathiazole and sulfadiazine.

EXPERIMENTAL

Forty-eight rabbits of 1800 to 2100 grams of weight were divided into four groups of 12 animals each. Each group was treated with one of the four compounds. The compounds were administered orally in capsules twice a day for

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ten days in succession (20 doses). The doses were given at 10 A.M. and 3 P.M. All compounds were administered in 4 different doses as follows:

I. Group	3 rabbits treated with sulfathiazole	0.05 per 1000 Gm. of body weight
	3 rabbits treated with sulfathiazole	0.1 per 1000 Gm. of body weight
	3 rabbits treated with sulfathiazole	0.2 per 1000 Gm. of body weight
	3 rabbits treated with sulfathiazole	0.25 per 1000 Gm. of body weight
II. Group	3 rabbits treated with sulfamerazine	0.05 per 1000 Gm. of body weight
	3 rabbits treated with sulfamerazine	0.1 per 1000 Gm. of body weight
	3 rabbits treated with sulfamerazine	0.2 per 1000 Gm. of body weight
	3 rabbits treated with sulfamerazine	0.25 per 1000 Gm. of body weight
III. Group	3 rabbits treated with sulfapyrazine	0.05 per 1000 Gm. of body weight
	3 rabbits treated with sulfapyrazine	0.1 per 1000 Gm. of body weight
	3 rabbits treated with sulfapyrazine	0.2 per 1000 Gm. of body weight
	3 rabbits treated with sulfapyrazine	0.25 per 1000 Gm. of body weight
IV. Group	3 rabbits treated with sulfadiazine	0.05 per 1000 Gm. of body weight
	3 rabbits treated with sulfadiazine	0.1 per 1000 Gm. of body weight
	3 rabbits treated with sulfadiazine	0.2 per 1000 Gm. of body weight
	3 rabbits treated with sulfadiazine	0.25 per 1000 Gm. of body weight

The weight of each animal was recorded twice weekly.

The urines were examined for blood and crystaluria during and after the period of drug administration. On the tenth day after cessation of drug administration the survivors were sacrificed. Autopsies were made on all rabbits which died during the experiment and on all sacrificed animals. Specimens of the tissues were fixed in 10 per cent formalin solution and stained by hematoxylin and eosin. In addition, deKossa's calcium stain was used in several cases according to Rake and co-workers,<sup>1</sup> Antopol, Lehr, and co-workers,<sup>4</sup> etc., who used this method for the determination of calcifying nephrosis in kidneys of animals which were damaged by sulfonamides.

Quantitative determinations of blood levels\* were made in a separate series of rabbits receiving the chemical compounds under investigation in doses of 0.05 and 0.1 Gm. per kilogram twice a day. The purpose of this comparative blood level study was to obtain an idea of the speed of excretion of the various compounds during and after treatment. The bloods were taken immediately before the first drug administration (10 A.M.) on the day following the eighth dose, and twenty-four hours after discontinuance of drug administration.

Under the circumstances of this investigation sulfathiazole showed the lowest blood levels, and sulfadiazine, sulfapyrazine, and sulfamerazine approximately the same low blood levels, only somewhat higher than sulfathiazole.

#### RESULTS

**Survivors.**—At the end of the experiment, 30 of 48 rabbits survived as follows:

- 8 survivors of 12 rabbits after treatment with sulfathiazole.
- 7 survivors of 12 rabbits after treatment with sulfadiazine.
- 7 survivors of 12 rabbits after treatment with sulfapyrazine.
- 8 survivors of 12 rabbits after treatment with sulfamerazine.

\*The blood levels were determined and various chemical compounds in this investigation supplied by the courtesy of Doctor G. W. Raiziss of the Dermatological Research Laboratories, Division of Abbott Laboratories, North Chicago, Ill., which is acknowledged with appreciation by the author.

All rabbits receiving doses of 0.05 or 0.1 Gm. per kilogram twice a day survived, remaining well, whatever compound was administered. At the dosage of 0.2 Gm. per kilogram twice a day none of the sulfadiazine rabbits survived, while one survivor was observed with sulfapyrazine, two with sulfathiazole, and two with sulfamerazine. The one of the two survivors given sulfamerazine showed extreme loss of weight, anorexia, weakness, and amotility at the end of the experiment. When doses of 0.25 per 1000 Gm. were given twice a day, none of the rabbits survived, except one of the sulfadiazine series. The deaths occurred from the fourth to the ninth day of drug administration except for one rabbit receiving sulfadiazine in dose of 0.2 per 1000 Gm., which died only four days after cessation of drug intake. The earliest deaths (on the fourth day of treatment) were observed with sulfadiazine (2 rabbits) and sulfamerazine (1 rabbit).

All rabbits dying during or after treatment showed rapid decrease of body weight, which became most evident when impairment of the urinary output indicated that severe kidney damage had been produced. Urinary block, with anuria during the last 36 hours before death, was observed with two rabbits of the sulfathiazole group and two of the sulfamerazine group, each animal receiving 0.25 per 1000 Gm. of drug twice a day (10 to 14 doses). The two animals of the sulfathiazole group showed amotility of the hind legs, tremor, and convulsions during the last 24 hours before death. Microscopic examination of specimens of the brains and spinal cords did not reveal pathologic changes. Hematuria was observed only microscopically in several rabbits of the various compound groups when the lethal dosage of 0.25 per 1000 Gm. twice a day had been used.

*Crystaluria.*—During drug administration crystaluria was observed with all four compounds when given for at least five to six days in doses of more than 0.05 per 1000 Gm. In the centrifugates of urine specimens collected over 18 to 20 hours significant crystal forms were seen which were decidedly different from all other crystals existing in the urines of normal rabbits. The number of these crystals apparently increased with higher doses of drug. After administration of sulfathiazole and sulfadiazine, we observed the characteristic crystals as described by Lehr and Antopol,<sup>11</sup> Prien and Frondel,<sup>12</sup> etc. After administration of sulfapyrazine, we found dark or muddy yellowish rhomboid-formed platelets, some of which showed indented outlines and globular forms. After sulfamerazine treatment, we observed crystals similar to the "shocks of wheat with eccentric binding" described by Lehr and Antopol<sup>10</sup> as crystals of acetyl-sulfadiazine.

*Appearance of Precipitate in Tissue.*—The precipitated particles found in the tubules consisted of amorphous or crystalloid material. Using high-power magnification (oil immersion) we never observed obvious crystalline forms except in sections from one rabbit dying after eight doses of 0.25 per 1000 Gm. of sulfathiazole. In this case we saw thin, glass-clear hexagonal platelets among amorphous masses of precipitate, the shape of which resembled the form of acetyl-sulfathiazole crystals photographed by Lehr and Antopol.<sup>11</sup>

#### AUTOPSIES

The pathologic changes of the organs observed at autopsy were largely confined to the kidneys, spleen, and liver, while the other organs generally

did not reveal significant pathologic changes. However, in one rabbit of the sulfamerazine group which died on the fifth day after eight doses of 0.25 Gm. of sulfamerazine per kilogram, extensive intestinal hemorrhages were found in the wall of the cecum. In this study concerning principally the nephrotoxic properties of the drugs tested, we are reporting in detail the findings only in both kidneys, liver, and spleen.

*Kidneys.*—In nearly all rabbits which survived the ten-day period of drug administration the appearance of the kidneys remained normal, when the dosage was not more than 0.5 or 0.1 Gm. per kilo twice a day. At that dosage only two survivors of the sulfathiazole series and one of the sulfamerazine series exhibited slight enlargement of one or both kidneys. In the rabbits which died after doses of 0.2 or 0.25 Gm. per kilo of the various compounds, the kidneys showed enlargement, edema, and hyperemia, the size varying from slight swelling to considerable enlargement up to twice the normal size. The color of these kidneys was either bluish-red or yellow-green, occasionally very pale, the consistency soft. Upon opening the kidneys, we found the medullar region congested, sometimes hemorrhagic; the cortical region usually pale, sometimes included in the dark red of the medullar region. Extreme enlargement was always associated with dilatation of the renal pelvis, and occasionally of the proximal part of one or both ureters. Pus was found in the dilated renal pelvis of two rabbits receiving doses of 0.2 Gm. of sulfadiazine per kilo, the one dying on the fifth day of administration, the other four days after discontinuance of the drug. In both animals hemorrhages were seen macroscopically in the medullar and cortical region; small particles of concretions were adherent to the walls of the renal pelvis. Gross concretions (uroliths) up to the size of a cherry stone were found in the dilated renal pelvis of five of the six rabbits receiving sulfamerazine in dose of 0.2 or 0.25 Gm. per kilo, four of which died during treatment. In some of these cases the concretions were large enough to obstruct the renal pelvis; small particles obstructed the mouth of one ureter also. In one rabbit, given 0.25 per 1000 Gm. doses of sulfamerazine, small blood clots were found in the renal pelvis. Also one of the two survivors given doses of 0.2 Gm. of sulfamerazine per kilo, when sacrificed, showed obstructing uroliths, while the other survivor did not exhibit extrarenal concretion. In each of the five sulfamerazine rabbits which had stones in the renal pelvis, gross particles of concretion were found embedded in the renal parenchyma also, with hemorrhages and tissue destruction around the foreign substance. This was also observed, although to a smaller extent, in the kidney of one rabbit dying after doses of 0.2 Gm. of sulfadiazine per kilo twice a day. Obviously in these cases, as Maisel and co-workers<sup>3</sup> described in dogs receiving sulfadiazine, parts of intratubular concretion had broken through the necrotic tubular walls so as to penetrate the surrounding organ tissue. Fig. 1 demonstrates the significant appearance of one kidney of a rabbit which received sulfamerazine and died on the seventh day (after 12 doses of 0.25 Gm. per kilo). Fig. 2 shows the form and size of the uroliths. The chemical analysis of crystals found in the kidneys of this rabbit and also the concretions found in the ureter disclosed the presence of the free and conjugated sulfamerazine. The conjugated material was several times larger than the free sulfamerazine.

At the same dosage of sulfapyrazine and sulfathiazole we found no gross concretions (uroliths) in the renal pelvis or ureters.

In several survivors given the lower doses of 0.05 or 0.1 Gm. of drug per kilo, permanent dilatation of the renal pelves and the proximal part of the ureters, but with no concretions present, was observed at the end of the experiment. This occurred in survivors of both the sulfathiazole and the sulfadiazine group, but in none of the rabbits surviving after sulfapyrazine administration in equal doses.

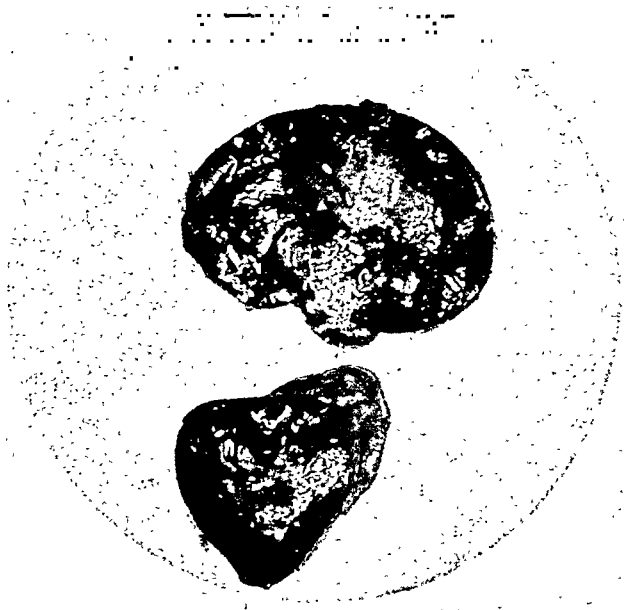


Fig. 1.

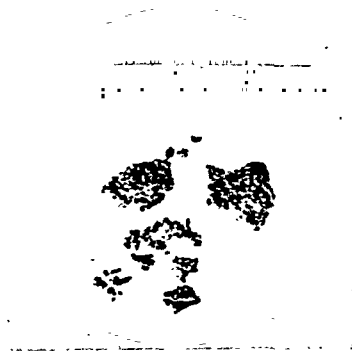


Fig. 2.

The *bladders* of all animals dying during treatment were examined. Only small amounts of urine were present or else the bladders were empty. Only in one of the sulfamerazine rabbits given 0.25 Gm. per kilo twice a day, some particles of concretions were found in the bladder; the urine present contained red blood cells.

Frequently marked differences were observed in the extent of injury of the two kidneys. While the one kidney at times showed severe change, the other showed only slight or moderate change.

*Spleen and liver* did not reveal gross changes from the normal when doses of 0.05 or 0.1 Gm. per kilo were used. After higher doses, namely 0.2 or 0.25 Gm. per kilo, changes in size, color, and consistency were observed frequently. When involved, the spleens were enlarged and hyperemic. Extremely small spleens were found in two rabbits, the one surviving sulfadiazine doses of 0.25 Gm., the other sulfamerazine doses of 0.2 Gm. per kilo. The livers, when involved, were enlarged, of a yellowish color, and of soft and fragile consistency.

#### MICROSCOPY

*Kidneys.*—The pathologic tissue changes produced in the kidneys of laboratory animals by prolonged sulfonamide administration present a characteristic appearance. Detailed descriptions have been published in the literature.<sup>1, 3-6</sup> Corresponding to the varying dosage, and in varying degree with the four compounds, we were able to observe the gradual development of the renal injury. The first evidence of injury was revealed in the cells of the lining epithelium of the convoluted tubules, the walls of which were exposed to the pressure of insoluble precipitate irritating and finally damaging the epithelial cells. In the afflicted tubules these cells appeared cloudy, swollen, granular or with small irregular vacuoles, the outline indistinct, the nuclei partly pale, partly fragmented; eventually the absence of the nuclei was encountered in many areas. Because of the accumulation of precipitate, the tubules were dilated, occasionally extremely dilated. Cellular and albuminous casts were seen in many tubules which had completely lost their epithelial lining; only cell debris and precipitate particles were found free in the tubules. Chiefly the convoluted tubules were injured; to a small extent also, Henle's tubules were at times involved, while the collecting tubules appeared either undamaged or only slightly affected, showing some cloudy swollen cells with indistinct outlines. Obvious lesions of the tubules usually were associated with dilatation of the glomerular spaces which sometimes contained a few precipitate particles. In rabbits which died after doses of 0.25 per 1000 Gm. of drug, we found extreme capsular dilatation, some glomeruli filling only a fifth or less of the space. Hemorrhages were found in the renal tissue of those animals in particular in cases where insoluble concretion had broken through the destroyed tubular walls, producing focal parenchymatous degeneration. In kidneys of rabbits which died after the first five days of drug intake, leucocytic infiltration was found to a varying extent around the damaged tubules.

In sections of rabbits which died during treatment extremely dilated tubules were found with no precipitate present. Obviously the crystalline masses had been partly removed by excretion before death; on the other hand, some precipitate may have been washed out during fixation of the specimens. We refer to the detailed descriptions by Rake et al.<sup>1</sup> in mice, Sobin et al.<sup>9</sup> in rats.

Our results summarized in Tables I and II refer to the extent, site, and degree of the pathologic changes which we observed in our sections. In every case both kidneys were examined microscopically. The symbols used indicate the degree of damage in the tissue observed with the various dosages of drug.

From the tables it will be observed that severe changes were found in all rabbits dying after the usually lethal dosage of 0.25 Gm. per kilo twice a day,

TABLE I

PATHOLOGIC TISSUE CHANGES OF THE KIDNEYS AFTER PROLONGED ADMINISTRATION OF SULFAPYRAZINE AND SULFAMETHYLDIAZINE (SULFAMERAZINE) AS COMPARED WITH SULFATHIAZOLE AND SULFADIAZINE

RABBIT NO.	DOSE PER KILO (GM.) TWICE PER DAY ORALLY	SULFATHIAZOLE				SULFADIAZINE			
		SUR-VIVORS (S)	DEATHS AFTER DAYS OF TREAT-MENT	DEGREE OF PATH-OLOGIC CHANGES OF KIDNEY TISSUE	ADDI-TIONAL REMARKS UROLITHS (U)	SUR-VIVORS (S)	DEATHS AFTER DAYS OF TREAT-MENT	DEGREE OF PATH-OLOGIC CHANGES OF KIDNEY TISSUE	ADDI-TIONAL REMARKS UROLITHS (U)
1	0.05	S	--	0	--	S	--	0	--
2	0.05	S	--	+	--	S	--	+	--
3	0.05	S	--	0	--	S	--	+	--
4	0.10	S	--	0	--	S	--	+	--
5	0.10	S	--	0	--	S	--	0	--
6	0.10	S	--	0	--	S	--	0	--
7	0.20	S	--	+	--	--	10	+++	Pyelitis and U
8	0.20	S	--	0	--	--	3	++	--
9	0.20	--	5	++	--	--	4	+++	Pyelitis and U
10	0.25	--	4	++	--	--	3	+++	--
11	0.25	--	5	+++	--	--	5	+++	--
12	0.25	--	6	++	--	S	--	++	--

0 indicates: no gross or microscopical changes from the normal.

+ indicates: dilatation of collecting tubules limited to small areas, with scarce foci of epithelial necrosis, occasionally with small amounts of precipitate free in the tubules.

++ considerable dilatation of convoluted and collecting tubules with focal necrosis of the lining epithellum, precipitate accumulated in the tubules, and cellular casts, some hemorrhages; in some areas dilatation of glomerular spaces.

+++ indicates: extreme dilatation of the glomerular spaces and of the convoluted and collecting tubules showing extensive epithelial necrosis, immense amounts of precipitate or gross concretions (uroliths), hemorrhages, occasional leucocytic infiltration around the afflicted tubules, and presence of numerous albuminous and cellular casts.

TABLE II

RABBIT NO.	DOSE PER KILO (GM.) TWICE PER DAY ORALLY	SULFAPYRAZINE				SULFAMERAZINE			
		SUR-VIVORS (S)	DEATHS AFTER DAYS OF TREAT-MENT	DEGREE OF PATH-OLOGIC CHANGES OF KIDNEY TISSUE	ADDI-TIONAL REMARKS UROLITHS (U)	SUR-VIVORS (S)	DEATHS AFTER DAYS OF TREAT-MENT	DEGREE OF PATH-OLOGIC CHANGES OF KIDNEY TISSUE	ADDI-TIONAL REMARKS UROLITHS (U)
1	0.05	S	--	0	--	S	--	0	--
2	0.05	S	--	0	--	S	--	0	--
3	0.05	S	--	0	--	S	--	+	--
4	0.10	S	--	0	--	S	--	+	--
5	0.10	S	--	+	--	S	--	+	--
6	0.10	S	--	+	--	S	--	+	--
7	0.20	--	7	++	--	S	--	+	--
8	0.20	S	--	+	--	S	--	+++	U
9	0.20	--	8	++	--	--	7	+++	U
10	0.25	--	4	+++	--	--	4	+++	U
11	0.25	--	5	++	--	--	3	+++	U
12	0.25	--	6	+++	--	--	6	+++	U



sulfamerazine producing the highest injury. Marked differences concerning the various compounds became evident when the sublethal dosage of 0.2 Gm. per kilo had been used. At this dosage the sulfathiazole group exhibited obviously less tissue damage than did the other compound groups, and the sulfapyrazine group less than did sulfamerazine and sulfadiazine. After use of the lower doses, i.e., 0.05 or 0.1 Gm. per kilo twice a day, no change from the normal was observed with any compound, except for an occasional focus of epithelial irritation or degeneration in a few convoluted tubules. Finally, it has to be noted that also in one of both survivors of the sulfathiazole group receiving 0.2 Gm. per kilo twice a day, no pathologic change was revealed at the end of the experiment; the other survivor of this group and one survivor of both the corresponding sulfapyrazine and sulfamerazine groups exhibited only slight pathologic change.

*Calcium deposits* were revealed by deKossa's stain in the kidneys of all rabbits receiving drug in doses of 0.2 or 0.25 Gm. per kilo for at least seven days. At this dosage we observed the beginning of calcium deposit upon necrotic parts of the tubular epithelium or around cell debris and precipitate particles free in the tubules. However, after administration of sulfathiazole, sulfapyrazine, and sulfadiazine, only slight calcium deposits were found in the cortical region. More advanced calcification became evident only in specimens of two rabbits receiving doses of 0.2 Gm. of sulfamerazine per kilo, the one dying after seven days of drug intake, the other when sacrificed.

*Spleen and Liver.*—When doses of 0.05 or 0.1 Gm. per kilo twice a day were used, no pathologic changes were observed with any of our compounds. At the dosages of 0.2 or 0.25 Gm. per kilo damage was observed with all compounds. When involved, the *spleens* showed marked changes limited to the Malpighian corpuscles with considerable to extreme reduction of the lymphocytes of the mantle zone and hyperplasia or, in some areas, complete disappearance of the germinal centers. Complete reduction of the lymphocytes of the mantle and marginal zones, with nothing remaining but collapsed stroma and a few pyknotic cells, was seen in the spleens of rabbits which died at the dosage of 0.25 per 1000 Gm. of the four drugs, and in two rabbits given 0.2 per 1000 Gm. of drug twice a day, the one belonging to the sulfapyrazine, the other to the sulfamerazine group.

The *livers*, when involved, showed vacuolization or complete degeneration of the peripheral liver cells of some lobules. The greatest development of liver cell damage was found in cases where obstruction of the renal passages had impaired the excretory function. Severe changes in spleen and liver obviously corresponded to the degree of kidney injury.

#### DISCUSSION

The nephrotoxic properties of sulfonamide derivatives are due principally to a mechanical effect of the insoluble drug precipitate which irritates and finally destroys the lining epithelium of the tubules by pressure necrosis, and simultaneously obstructs the renal passages.<sup>6, 7, 9\*</sup> The increase in precipitate masses which cannot be excreted as rapidly as formed in the convoluted tubules

\*This fact does not exclude the possibility that after chronic administration of generally toxic doses the mechanical injury may be associated sometimes with a direct toxic effect on the parenchyma of the kidneys.

suppresses the urinary output and, as a result, may cause urinary block. This fatal symptom is most common when gross uroliths have been formed in the renal pelves barring the urinary passage. In this respect the varying solubility of the drugs and their acetylated products is of great importance. For instance, poor solubility combined with slow excretion is a factor which can favor the formation of gross concretions (uroliths).

For this reason, one can expect with justification that rabbits, which have a great ability to acetylate sulfonamide derivatives, will be more apt to have gross concretions in the kidneys after prolonged administration of sulfonamides, which form extremely insoluble acetylated products. Insofar as the relationship between precipitation and solubility is concerned, fewer gross concretions (uroliths) are to be expected in rabbits after prolonged administration of sulfonamide derivatives, which have acetylated products that are more soluble.

As reported by Hamburger, Rueggsegger, et al.<sup>15</sup> on sulfapyrazine and sulfadiazine, and by Welch, Mattis, et al.<sup>21</sup> on sulfadiazine and sulfamethyldiazine (sulfamerazine), the acetylated derivatives of these compounds proved to be more soluble than the unacetylated drug; and, as to sulfamerazine, both sulfamerazine and its acetylated products proved to be somewhat more soluble than the respective sulfadiazine (in urine at a pH of 7 or less); furthermore, the degree of acetylation was the same with sulfamerazine as it was with sulfadiazine.<sup>21-23</sup> From their experience with animals and human beings and their results in comparative blood level studies, Welch and his group concluded that sulfamerazine is more rapidly and more completely absorbed from the gastrointestinal tract and yet more slowly eliminated by the kidneys than is sulfadiazine at equal dosage. Despite their observation that the blood reaching the kidney was very much lower in its sulfadiazine content than it was in sulfamerazine content, these authors observed that more sulfadiazine than sulfamerazine was excreted in the urine during the first twenty-four hours. These investigators used various species of laboratory animals in their experiments, i.e., mice, rats, chickens, dogs, and monkeys. In our comparative study on kidney damage following prolonged drug administration, we used rabbits only. Under the conditions of our experiment we observed that, insofar as renal precipitation is related to solubility, sulfamerazine apparently is able to produce more kidney damage in rabbits than do sulfadiazine, sulfapyrazine, and sulfathiazole at equal dosage, because of the great ability of sulfamethyldiazine to form intrarenal and extrarenal concretions in rabbit kidneys. This was observed with five of six rabbits given 0.2 or 0.25 Gm. of sulfamerazine per kilo twice a day, in comparison with two of six rabbits which were given sulfadiazine in equal doses. The two rabbits of the sulfadiazine series showed only a few small particles of concretion in the renal pelves. Neither with sulfapyrazine nor with sulfathiazole did we observe the formation of uroliths deposited in the renal pelves of rabbits.

In evaluating the extent and degree of the kidney injury observed under the conditions of our experiment we had to consider not only the site and extent of organ changes found in rabbits which died during drug administration, but also the permanent tissue change observed in the sacrificed survivors.

When we sacrificed the rabbits on the tenth day after, <sup>the last</sup> dose of 0.05 or 0.1 Gm. per kilogram of drug, we found <sup>the path-</sup>

ologic change in the kidneys except for an occasional focus of tubular epithelial necrosis. When doses of 0.2 Gm. per kilogram were used, sulfathiazole apparently caused less tissue changes in the kidneys of the surviving animals than did the other compounds. The one survivor receiving doses of 0.2 Gm. of sulfapyrazine per kilo and the one of the two survivors treated with equal doses of sulfamerazine showed lasting kidney damage of only a small degree, while the other sulfamerazine survivor exhibited severe kidney damage, as we described. At equal dosage, there were no survivors in the corresponding sulfadiazine group. Surprisingly, one rabbit receiving the highest dosage of sulfadiazine, namely 0.25 Gm. per kilo, survived, showing only a moderate extent of tissue change; this was the only one of all rabbits used for our experiment which survived the ten-day administration of 0.25 Gm. of drug per kilogram twice a day.

The deposit of *calcium*, as revealed by deKossa's calcium stain, cannot be considered as specific for sulfonamide injury, but only as a "general response of the kidneys to injury" (Sobin and co-workers"). The demonstration of calcification, however, has been generally acknowledged as an index of permanent tissue damage insofar as calcium deposit usually corresponds to the extent and site of necrotic cell substance or other foreign material existing in the organ tissue.

The presence of sulfonamide *crystals*, which we found in the urines of our rabbits during drug administration, did not give any indication of injury as long as the output of urine appeared normal and the urine did not show blood content macroscopically or microscopically.

The pathologic changes observed in *spleen* and *liver* generally corresponded to the degree of impairment of the renal function. The accumulation of toxic amounts of drug in the blood and liver may be responsible for the pathologic tissue changes observed particularly in the livers.

For this comparative study we considered only the nephrotoxic effect after prolonged drug administration as the basis for evaluation of the compounds. Since the number of animals used for our investigation was limited to three rabbits for each dosage, we do not feel justified in basing conclusions concerning the toxicity on the number of survivors.

#### CONCLUSIONS

Under the conditions of our experiment, sulfapyrazine and sulfamethyldiazine (sulfamerazine), when given in doses of not more than 0.05 or 0.1 Gm. per kilogram twice a day for ten days in succession (20 doses), did not produce marked tissue damage in the kidneys of rabbits, except for an occasional focus of tubular epithelial necrosis in a few areas. The same results were obtained with sulfathiazole and sulfadiazine. All rabbits in our experiments receiving doses of 0.05 or 0.1 Gm. per kilogram survived.

When doses of 0.2 or 0.25 Gm. per kilogram were given, all compounds showed nephrotoxic effect in varying degrees.

Sulfathiazole in dosage of 0.2 Gm. per kilogram produced a lesser degree and lesser extent of kidney damage than did the other compounds. Sulfapyrazine produced less kidney damage than did sulfadiazine or sulfamerazine.

## CONTROL MEASURES

Progress in therapy was judged by the clinical response; namely, an abatement of the headache, a fall in temperature, freedom from nausea and vomiting, and lessening of the neck rigidity. The blood sulfadiazine level was used as an index of the intensity of therapy. No effort was made to maintain a fixed level as it was found that neither the exact height nor constancy of the level run parallel with clinical improvement or bacteriostatic response.

Patients severely ill often showed as marked a clinical improvement and bacteriostatic response when the sulfadiazine level was maintained at 5 mg. per 100 c.c. as at 15 mg. per 100 c.c. This is illustrated by Table II, in which, with a blood sulfadiazine level of 7.05 during the second twenty-four-hour period of the disease, the spinal fluid smears and cultures were negative and the clinical signs disappeared or regressed. This is further emphasized by Table I which represents the sulfadiazine level at the end of the first twenty-four-hour period in 4 patients who ran a parallel course, and who showed about the same clinical improvement and bacteriostatic response.

TABLE I

VARIATIONS IN THE BLOOD SULFADIAZINE LEVELS IN PATIENTS SHOWING ABOUT THE SAME CLINICAL IMPROVEMENT AND BACTERIOSTATIC RESPONSE

NAME	GRAMS SULFADIAZINE FIRST 24 HOURS	SULFADIAZINE BLOOD LEVEL AFTER FIRST 24 HOURS
R. S.	11.0	8.4 mg. per 100 c.c.
O. C.	11.0	26.9 mg. per 100 c.c.
J. M.	11.0	10.6 mg. per 100 c.c.
C. B.	11.0	2.5 mg. per 100 c.c.

Sulfadiazine blood level determinations were done every other day, or oftener, depending upon the severity of the cases.

TABLE II

FINDINGS IN ONE PATIENT WHICH MAY BE TAKEN AS REPRESENTATIVE OF THE GROUP

		ADMISSION	FIRST 24-HR. PERIOD	SECOND 24- HR. PERIOD	THIRD 24-HR. PERIOD
Spinal Fluid Findings	Appearance	Cloudy	Turbid	Opalescent	Clear
	W. B. C.	5250	4710	1010	452
	Neutrophiles per cent	67	91	94	15
	Lymphocytes per cent	33	9	6	85
	Smear	+	+	0	0
	Culture	+	0	0	0
	Sugar in mg. per 100 c.c.	Trace	33.0	37.0	50
	Sulfadiazine level in mg. per 100 c.c.	0	4.4	6.0	12.7
Clinical Signs	Headache	+++	±	0	0
	Neck stiffness	+	+	±	0
	Rash	+	±	0	0
	Kernig's sign	0	0	0	0
Sulfadiazine	Total amount of sulfadiazine given in each 24-hour period	0	7 Gm.	6 Gm.	6 Gm.
	Grand total in the three 24-hour periods				19 Gm.
	Sulfadiazine level in mg. per 100 c.c.		8.8	7.50	15.0

Spinal punctures were done every twenty-four hours in some of our patients as a matter of interest to determine the effects of therapy. The record of the findings in one of our patients, as shown in Table II, is representative of the group.

The fluid intake and output were measured each day. Urinalyses were done every other day with an eye to the presence of albumin, red blood cells, and sulfadiazine crystals. A white blood count and hemoglobin determination were made every other day, as an index of a toxic effect on the bone marrow.

Any tendency to neutropenia or impaired kidney function was met by a reduction in the dosage of the drug and by venoclysis.

#### DRUG COMPLICATIONS

Two of our patients had hematuria and some nitrogen retention. One of these patients also had an oliguria. Another patient developed drug fever. In one of our fulminating cases, where the blood sulfadiazine level was pushed to 29 mg. per 100 c.c., the urinary output dropped to 450 c.c. during the twenty-four-hour period preceding his death, during which period he had received 2500 c.c. of isotonic salt solution parenterally. On the day preceding, his urinary output was 1450 c.c. with a total fluid intake of 4000 c.c. There were some amorphous deposits in the bladder, ureters, and kidneys, and a blocking of some of the tubules.

#### SUMMARY AND CONCLUSIONS

Of the 41 patients observed with a meningococcus infection, 33 had pus cells and organisms in the spinal fluid; 2 with a positive blood culture had pus cells, but no organisms in the spinal fluid; and 6 had a completely negative spinal fluid, but a positive blood culture.

The presence of headache, weakness and instability, vomiting, stiffness of the neck and a skin eruption were the five earliest signs present and were considered to be evidence presumptive of the presence of meningococcus infection. The presence of headache with any two of the other findings was considered an indication for spinal puncture.

Treatment was started immediately after spinal puncture without awaiting laboratory reports. Progress was determined by clinical response. In the management of these cases limited dependence was placed on the blood sulfadiazine level.

The mortality rate for the entire series was 5 per cent.

## THE ASSOCIATION OF ACUTE INTERSTITIAL PANCREATITIS WITH ACUTE PNEUMOCOCCIC MURAL ENDOCARDITIS\*

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THE coexistence of two clinical entities is not uncommon, but where the clinical and pathologic entities are of unusual occurrence and very difficult of diagnosis, such a case, we feel, merits recording. It is with this view in mind that we wish to report the following case:

M. B., 47, housewife, was admitted to Medical Service 2 at the Mount Sinai Hospital on the fifth of July, 1938, with the following history. On the 18th of June she was seized with severe abdominal pain centering in the epigastrium, radiating to both hypochondrial regions, and associated with nausea, vomiting, and fever. She was sent to the Philadelphia General Hospital where she was studied for a period of ten days. Studies there revealed a leucopenia on three blood examinations, sterile blood culture, and normal blood chemistry findings. Two roentgenograms suggested a pneumonic lesion on the right side. X-ray of the gastrointestinal tract was negative. No definite diagnosis was established. The symptoms persisted. She insisted on being transferred to the Mount Sinai Hospital where she had been treated for a pleuropneumonia of the right lung five years previously.

On admission, she appeared very ill, pale, restless, somewhat dyspneic, and markedly dehydrated. There was evidence of an acute upper respiratory infection, harsh and prolonged expirations were heard, and impaired resonance with showers of crackling râles were detected over the right lower lobe. The heart was rapid but presented no other abnormalities. The abdomen was distended, tender, and tympanitic. Peristalsis was audible. Vomiting was one of the most annoying of her symptoms. Blood pressures were 120/85; temperature, 101° F.; pulse, 110; respirations, 36. Because of the predominance of the abdominal symptoms and signs, a provisional diagnosis of an "acute abdomen," most likely pancreatitis complicated by pneumonitis, was made. She was given fluids and electrolytes by hypodermoclysis and studies were undertaken.

The hemogram revealed a hemoglobin of 60 per cent (Sahli), 8.7 Gm.; red blood cells, 3,500,000; white blood cells, 11,200, of which 84 per cent were polymorphonuclear neutrophils and of which 47 per cent were young forms. Blood platelets numbered 240,000. Blood cultures were sterile. Blood urea nitrogen was 13 mg.; blood cholesterol, 182 mg.; blood sugar, 145 mg. per 100 c.c. of blood; urinary diastase present in dilution, 1:80; serum lipase reading was 0.85 c.c., icteric index, 8.4 units; urea clearance, 58 per cent. The urine showed a trace of protein. Serology was negative. The sputum contained no pneumococci. Staphylococci and nonhemolytic streptococci were present. X-ray of the chest was negative.

Within a few days the abdominal symptoms subsided and the patient felt much better. However, within three days, toxicity again became manifest; pyrexia assumed a high level, with pulse and respirations in proportion. Within ten days a palpable mass was felt in the left hypochondrium. This was considered to be an enlarged spleen, although the possibility of an enlarged kidney was also considered. This enlargement occurred after a blood transfusion. The blood picture remained the same. The possibilities of typhoid, submiliary tuberculosis, and brucellosis were considered. Agglutination tests of the blood, urine,

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and feces examinations suggested no specific diagnosis. Intravenous urography revealed a normally sized and placed kidney with a suggestion of dilatation of the calices in the right kidney. X-ray of the lungs revealed congestion but no consolidation. The hemograms on 7/19/38, a day before death, revealed a leucocytosis of 29,100 with 90 per cent polymorphonuclear neutrophils, of which 82 per cent were segmented. The blood urea nitrogen at that time was 15 mg. per 100 c.c. of blood. In spite of transfusion and general care the toxicity continued. Her temperature reached 105° to 106° F. Signs of meningeal irritation appeared as manifested by nuchal rigidity. She died on 7/20/38 before a spinal puncture could be done, fifteen days after admission.

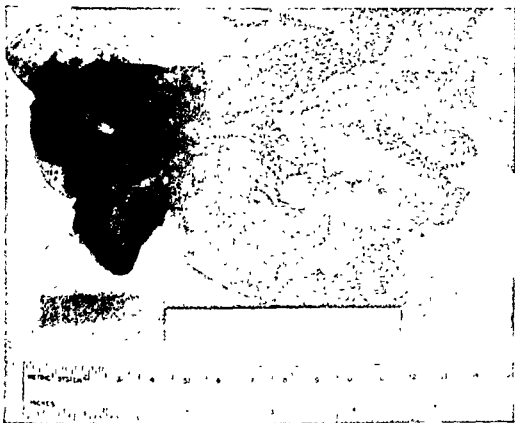


Fig. 1.—Heart showing mural thrombus. (Section taken through site of thrombus)

Pathologically the underlying finding at autopsy was a pneumococcic Type III septicemia. Abscesses from which this organism was isolated were present in the lungs, heart, spleen, kidney, liver, and perirenal fat tissue. In addition to these findings, three totally unexpected changes were observed at autopsy, the gross pathologic findings in the spleen and heart and the microscopic findings in the pancreas. During life, as already stated, a mass was palpated in the left upper quadrant. This proved at autopsy to be a spleen roughly three times normal size, whose surface was covered with fibrinous exudate. The enlargement of the organ was largely due to the replacement of the midportion of the spleen by a large cavity filled with broken-down splenic tissue and blood. The lining of this pseudocyst was ragged and histologically consisted of broken-down splenic tissue. Several of the branches of the splenic artery were thrombosed. In the absence of a more satisfactory explanation, we believe that these splenic changes were caused either by the rupture of an intrasplenic vessel or by hemorrhagic liquefaction of an area of early infarction.

The most unexpected finding was the presence of a mural thrombus involving the left auricle, slightly beyond the line of attachment of the posterior mitral leaflet (Fig. 1). The vegetation covered about 1.5 cm. of the auricular wall. It was raised but flat and overlaid an auricular wall honeycombed throughout almost its entire thickness by a suppurative process. The mitral valve leaflets themselves were moderately thickened but not deformed. Histologically there were no evidences of an active inflammatory process in the valve leaflets. The pathogenesis of this vegetative process becomes a matter for pathologic speculation. The heart muscle showed histologically an active acute diffuse and focal suppurative process. It is probable that the suppurative process in the region of the thrombus had destroyed surface myocardial cells and provided the setting for the thrombus.

The third unexpected finding was the presence of pancreatic changes. The pancreas shows diffuse edema (Fig. 2). In many regions there was also small focal infiltration, and in other areas more diffuse infiltration with polymorphonuclear cells and lymphocytes (Fig. 3). In some areas the infiltration was predominantly lymphocytes. In still other such regions fibrous tissue cells were present in increased numbers.

In addition, the lungs presented multiple abscesses, one large abscess in the left upper lobe and in some areas an unresolved pneumonic process. The kidneys showed widespread suppuration. The brain showed a diffuse suppurative meningitis, particularly marked in the basal regions. The cerebrospinal fluid was turbid and contained flocculi. A pneumococcus Type III was obtained on culture of the meningeal exudate.

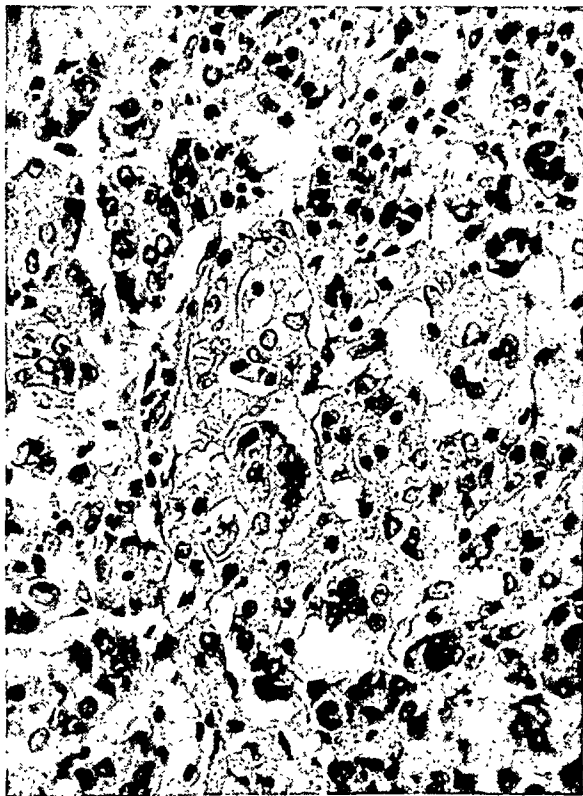


Fig. 2.—Pancreas—diffuse edema.  $\times 400$ .

#### DISCUSSION

The incidence of acute pneumococcic endocarditis as reported in the literature varies from 0.06 to 2 per cent. Ruegsegger<sup>1</sup> reports an incidence of 2.9 per cent of endocarditis in her series of acute pneumococcic infections.

Pneumococcic endocarditis may complicate pneumonia at any stage of the illness or days or weeks after an apparently uneventful convalescence. Six of Ruegsegger's patients were afebrile from two to seven days when the complication manifested itself by sudden chills and fever. It differs from subacute bacterial endocarditis in the following ways:

1. It affects older age groups.
2. It may involve a perfectly normal endocardium.



3. Superficial embolism is very rare, but abscess embolism, usually purulent in character, is common.
4. Purulent meningitis is a frequent complication.
5. The course of the disease is rapid. Death takes place within four or five weeks.



Fig. 3.—Pancreas—diffuse infiltration with polymorphonuclear white blood cells.  $\times 400$ .

Mural endocarditis has been reported, but it is much less common than the valvular types. In Ruegsegger's series only three were noted and even in these the process extended from the valves onto the mural endocardium. Acute bacterial endocarditis is a part of an overwhelming general infection, so that the septicemia overshadows the endocardial manifestation. This is particularly so where the pathology is mural in type. Hence the diagnosis is usually made at post mortem.

Acute interstitial or acute edematous pancreatitis is a definite clinical entity. It runs a milder course than that of acute pancreatic necrosis and recoveries are the rule.

Elman's<sup>2</sup> description of the histologic picture of this disease is worth quoting. He writes: "The striking finding was the marked infiltration of acute (polymorphonuclear) inflammatory cells into the interstitial tissue between lobules as well as acini, often with evidence of edema as shown by the outpouring of fluid." In another publication<sup>3</sup> two years later he expresses the same

opinion. Cole's<sup>4</sup> description of this condition coincides with that of Elman. The pathologic findings justify our clinical diagnosis in this case. One may ask whether the pancreatic lesion is not part of the generalized infection. It seems to us that the nature of the pathologic process in the pancreas, i.e., the absence of any gross suppurative process, would probably speak against such a possibility.

If one is justified in speculation, and one is frequently compelled to speculate in clinical medicine, there is a strong possibility that the acute pancreatitis was the initial pathologic lesion. The suddenness of the onset with predominantly abdominal symptoms and the abdominal signs are typical of acute pancreatitis. While it is true that pneumonia may be ushered in with abdominal signs, the severity and persistence for almost two weeks would rather speak against such a possibility. It is logical to consider that the lowered resistance caused by starvation and dehydration due to the pancreatitis prepared the soil for the invasion of the Type III pneumococcus with its subsequent multiple suppurative lesions. One of Elman's autopsy reports is from a patient who died from another cause some weeks after the attack of acute pancreatitis.

This case possesses the criteria differentiating acute bacterial from subacute bacterial endocarditis. She was 47 years old, there was no evidence of any previous heart disease, and the emboli were limited to the viscera and were suppurative in nature. She had purulent meningitis, and the entire course of her illness was about 5 to 6 weeks.

The pneumonia was of the bronchial type as evidenced by the limited physical signs and x-ray findings. Multiple abscesses are more common in this latter type than in pure lobar pneumonia. The rather large size of the abscess in the left upper lobe makes it a likely source of the septicemia involving the endocardium as well as the other viscera.

#### SUMMARY

1. A case is reported presenting the association of two rare clinical pathologic entities, viz., acute bacterial mural endocarditis and acute interstitial pancreatitis.

2. The likelihood of the interstitial pancreatitis being the primary process is suggested.

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## NODAL RHYTHM AND BUNDLE BRANCH BLOCK FOLLOWING ASPIRIN HYPERSENSITIVITY\*

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DRUG sensitivity is not uncommon, and as aspirin is a favorite drug, one would expect to see an occasional abnormal reaction after its ingestion, but Gardner and Blanton<sup>1</sup> have shown that although enormous quantities of aspirin are consumed every year in the United States, only a rare case of severe hypersensitivity has been reported.

Skin rash and gastrointestinal disorders are the common sequelae to drug overdosage; severe anaphylactic manifestations, such as dyspnea, cyanosis, asphyxia, generalized circulatory collapse, convulsions or profuse urticaria, are very unusual. Wilcox and Andrus,<sup>2</sup> in animal experiments on the isolated heart, have provoked anaphylactic reactions using horse serum, in which the electrocardiogram showed ectopic rhythms, PR prolongations, and QRS and T changes. These abnormalities were transient and frequently disappeared within fifteen minutes.

Our case represents an analogous situation in the human heart. The sensitizing mechanism was a drug rather than horse serum. Anaphylactic shock with transient nodal rhythm and bundle branch block occurred after the ingestion of five grains of aspirin. Within twenty-four hours all of these phenomena had disappeared.

### CASE REPORT

R. M., a white male, aged 50 years, was first seen in June, 1937, at the Medical College Hospital. He was brought to the hospital as an emergency, complaining of a sudden attack of breathlessness associated with pain in his right chest. He had suffered two previous attacks within a period of a month; both of these had subsided rather promptly after rest in bed for several hours. The pain in the chest was just to the right of the sternum at the fourth intercostal space and did not radiate. The examination at that time showed the blood pressure to be 135/85, temperature, 98.6° F., pulse, 80. An electrocardiogram was taken, but the only abnormality was a left axis deviation and a negative T wave in Lead III. The patient left the hospital two days after admission.

He was readmitted four years later in January, 1941, complaining again of breathlessness and pain in his right chest, but at this time it was associated with excessive lacrimation and a serous nasal discharge. The entire episode occurred after he had taken five grains of aspirin. The patient volunteered the information that he had noticed the same type of discomfort after taking aspirin in the past. The examination revealed a very weak male, bathed in perspiration, with chest pain which radiated to both arms. The skin was cold and clammy, pulse rate, 110, BP, 110/60, temperature, 100° F. An electrocardiogram was immediately taken, and this tracing showed a nodal rhythm and bundle branch block. The temperature subsided to normal in twenty-four hours. The urinalysis was negative, hemoglobin, 86 per cent Sahli, 4,900,000 erythrocytes, white blood cells, 13,400, with 89 polymor-

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phonuclear neutrophils, 6 lymphocytes, and 5 monocytes; the Wassermann was negative. The next day all of his symptoms had subsided. Another electrocardiogram showed that the nodal rhythm and bundle branch block had disappeared; the only unusual change in the tracing was a negative T-4. The patient remained in the hospital five days and then signed his release.

His third admission to the hospital was on March 12, 1943. At that time he returned for an elective operation on his nose. An electrocardiogram revealed the same contours as the original tracing in 1937. The chart shows the electrocardiographic changes.

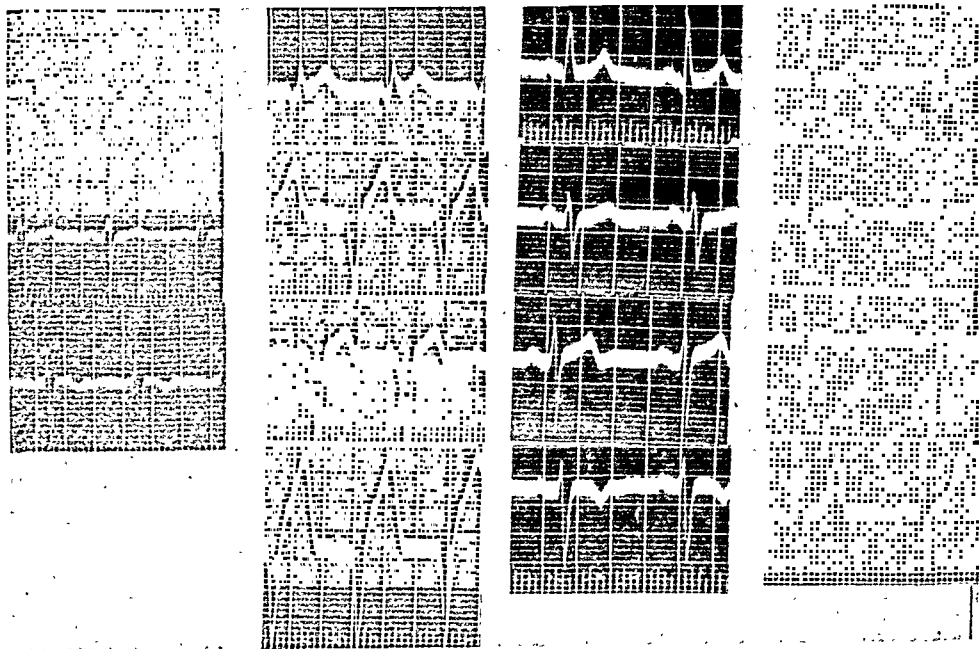


Fig. 1.—A, QRS—0.06; deep Q-1; deep S-2; negative P-3; deep S-3; negative T-3. Left axis deviation.

B, QRS—0.12; varying P-1; deep Q-1; slurred R-1; wide QS-2; high T-2; wide QS-3; high T-3; deep Q-4; high T-4. Nodal rhythm and left discordant bundle branch block, left axis deviation.

C, QRS—0.08; deep Q-1; deep S-2; deep S-3; diphasic T-3; negative T-4. Left axis deviation.

D, QRS—0.06; deep Q-1; deep S-2; negative P-3; deep S-3; negative T-3. Left axis deviation.

#### DISCUSSION

Although the original electrocardiogram taken in 1937 was not quite normal, the left axis deviation and T-3 negativity were not considered significant changes in this particular case, and after six years there were no added abnormalities in the electrocardiogram. Longcope<sup>3</sup> has demonstrated that there is a definite relationship between clinical serum disease and certain drug reactions. He considered the arsphenamines and sulfonamides the more dangerous drugs, but we have shown that aspirin may produce similar phenomena. Rich<sup>4</sup> studied the tissues of patients who had died after receiving horse or rabbit serum. The most interesting necropsy finding in these cases was an acute arteritis and periarteritis affecting the smaller vessels, and he concluded that vascular lesions of this type could be a manifestation of an anaphylactic form of hypersensitivity in man. We assume that this same type of reaction occurred in our case, but that it was transient and the patient was not left with any permanent damage of the smaller coronary vessels.

## CONCLUSION

Aspirin is an excellent analgesic and, considering its enormous consumption, only occasionally causes any severe reactions in human beings. This case is an example of very unusual sensitivity to the drug.

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## TRANSIENT T-WAVE INVERSION FOLLOWING PAROXYSMAL TACHYCARDIA\*

S. L. ZIMMERMAN, MAJOR, M.C., A.U.S.

**A**BNORMALITIES of T waves of the electrocardiogram following bouts of paroxysmal tachycardia, unassociated with myocardial infarction including T-wave flattening and inversion, have recently been reported.<sup>1, 2, 3, 6</sup> Their occurrence may follow either supraventricular tachycardias or, probably more often, those of ventricular origin. The inversion may persist a varying length of time following cessation of the paroxysm, even as long as two months, without necessarily entailing a diagnosis of infarction. Geiger<sup>2</sup> suggests that there may be some relationship between the length of the attack and the duration of the T-wave inversion. This, however, has not been the usual experience.

The importance of correct diagnosis in these cases, especially with reference to myocardial infarction, appears obvious. It is believed that the following three cases illustrate the evolution of these T-wave changes clearly, and it is felt that their significance should be more generally recognized. The first case is one of supraventricular tachycardia; the second and third are of the ventricular type.

### CASE REPORTS

CASE 1.—B. L., a 47-year-old white female, was admitted at 10:30 P.M. Aug. 4, 1943, with a paroxysmal tachycardia which began at 9 P.M. the same night. She gave a history of numerous attacks of rapid heart action, for a number of years, lasting from ten minutes to thirteen hours. During these attacks she had been cognizant of palpitation, but between them she was asymptomatic. No unequivocal history of a diminished cardiac reserve was obtained, and there was no history of retrosternal distress related to exertion. Her activities had been markedly restricted because of them. She had taken quinidine sulfate on occasions, and this drug had been quite effective in diminishing the number of paroxysms, as well as terminating them, once they had begun. She had chorea as a child, following an attack of tonsillitis. The remaining history was not relevant. Examination disclosed a 47-year-old white female, somewhat flushed, in the semiorthopneic position, with a regular ventricular rate of 200 to 210 per minute. The blood pressure was 130/110. The fundi revealed a mild retinal arteriosclerosis. The heart was not enlarged. There were no thrills, shocks, or abnormal thrusts. No murmurs were heard. The radial vessels were thickened. There were no evidences of congestive failure. The remaining examination was irrelevant. The electrocardiogram revealed a supraventricular tachycardia, with a rate of 210 per minute. (Fig. 1*d*.)

Orbital pressure, carotid sinus pressure, and vomiting resulted in some gradual slowing, but the rapid rate soon ensued, following cessation of stimulation. She was given dilaudid HCl  $\frac{1}{32}$  grain. She had stated that she was sensitive to morphine, and was reassured, but her arrhythmia continued. At 12:55 A.M. Aug. 5, 1943, she was given 20 mg. of mechlolyl. There was an intense vasodilatation, especially of the face and chest. The blood pressure fell to 112/90, but the rate remained unchanged. Carotid sinus pressure following mechlolyl failed to alter the arrhythmia.

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Quinidine sulfate in doses of six grains at 1 A.M. and six more grains at 6 A.M. was followed by an abrupt change in rate at 8:20 A.M. the morning of August 5. The paroxysm lasted eleven hours and twenty minutes. A second electrocardiogram (Fig 1B) showed a regular sinus rhythm with a rate slightly over 100, an inverted T-1 and T-4, and a flattened S-T interval with a shallow T wave in Lead II. The quinidine sulfate was continued in doses of three grains four times daily, and on Aug 7, 1943, electrocardiogram (Fig 1C) was obtained. The T waves in Leads I, II, and IV *F* reverted to an upright position. The patient was discharged and she resumed her usual activities immediately.

LEAD 1.

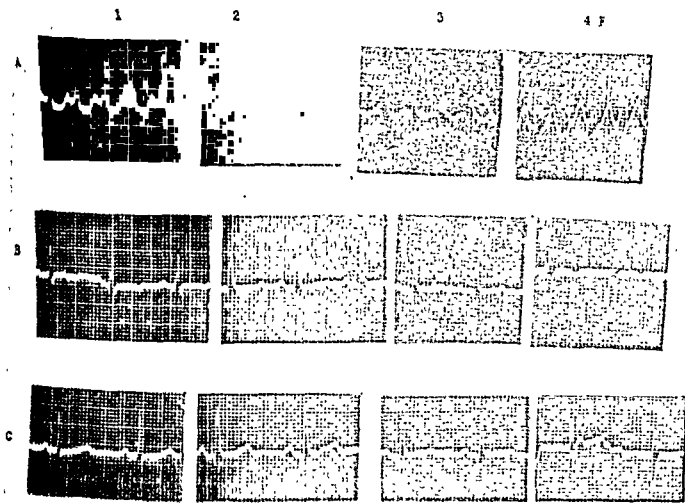


Fig. 1.

*Comment.*—A paroxysm of supraventricular tachycardia of eleven hours and twenty minutes' duration, followed by transient inversion of the T waves in Leads I and IV *F*, treated with sedation, vagal stimulation, and quinidine is presented. Two days following cessation of the arrhythmia, the T waves had reverted to their normal upright contour, in spite of continuation of the quinidine salt. There was no clinical or electrocardiographic evidence of organic heart disease, although the patient had a mild hypertension and a moderate degree of retinal and peripheral arteriosclerosis. That there was no myocardial infarction was obvious from the clinical course, the rapid reversion of the T-wave changes, and the absence of any S-T elevation and Q waves.

CASE 2.—B. W. M., a 46-year-old white male, was first admitted Feb. 12, 1939, complaining of nausea and a rapid, "fluttering" heart action, with sudden onset, the day prior to admission, accompanied by shortness of breath. There was a history of exertional dyspnea but none of anginal syndrome. Other than the above, his health had been considered good. There was no history of antecedent hypertension, syphilis, or rheumatic fever.

On admission, he was cyanotic and moderately dyspneic. The ventricular rate was 216 per minute and quite regular. The pulses were barely perceptible. The blood pressure was 90/70. There was no apparent enlargement. No murmurs were heard. The peripheral vessels

were sclerotic. There was no evidence of congestive failure. The remaining findings were not relevant. Carotid sinus pressure failed to alter the rate significantly. The electrocardiogram revealed a ventricular tachycardia (Fig. 2 A).

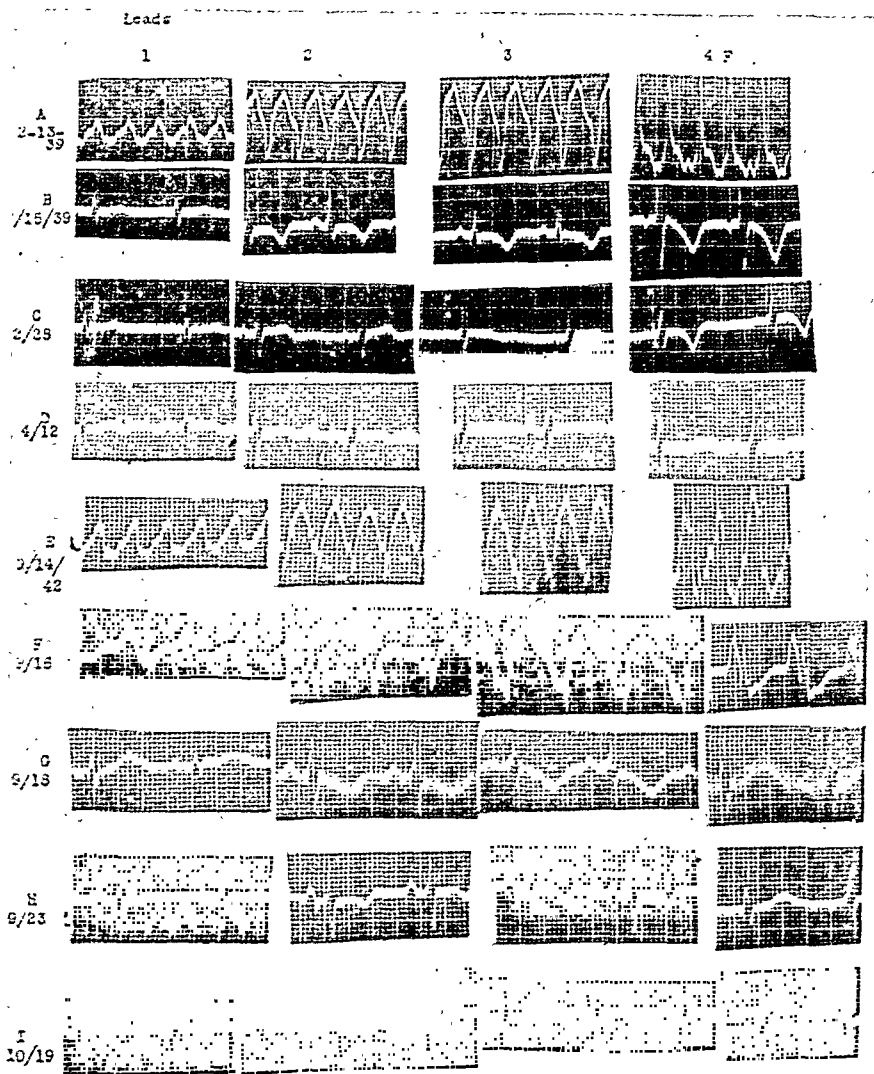


Fig. 2.

*Course.*—The patient remained afebrile throughout his hospital stay. No friction rub was audible. He was given small doses of digitalis, and the sinus rhythm became re-established on Feb. 15, 1939, the paroxysm lasting about four and a half days. Electrocardiogram (Fig. 2B), obtained Feb. 15, 1939, revealed marked inversion of the T waves in Leads II, III, and IV F, with depression of the S-T junction in Leads II, III, and possibly IV F. No quinidine was administered, but digitalis was continued in small daily doses, the patient probably having never been fully digitalized. The electrocardiogram of February 28 revealed a normal sinus rhythm, a diphasic plus-minus T-2 and T-3, and a deeply inverted T-4 (Fig. 2C), the rhythm continuing regular sinus. His course continued uneventful, and the last electrocardiogram during this admission (Fig. 2D) revealed an upright T-2, a very shallow, probably diphasic, plus-minus T-3, and a shallow, inverted T-4. The U waves were prominent in all leads. The patient was discharged as having attained maximum benefit, Apr. 16, 1939.



On the second admission, Sept. 14, 1942, he reentered with an attack of paroxysmal tachycardia of 48 hours' duration, which had not responded to digitalis or quinidine administered by his physician. He was excited, apprehensive, dyspneic, and somewhat cyanotic. The hands were cold and clammy. The ventricular rate was 208 and regular. The blood pressure was unobtainable. There were no murmurs and the heart was not enlarged. There was no evidence of congestive failure. The remaining findings were not consequential. Further history this time revealed the fact that he had had numerous attacks of tachycardia since 1929, lasting from a few hours to a few days. Electrocardiogram (Fig. 2F) was obtained on admission.

**Course.**—The patient was afebrile throughout his hospital stay. No friction rub developed. He complained of no pain. Large doses of quinidine sulfate were started orally on the day of admission, augmented with prostigmine. At 9 A.M., Sept. 16, 1942, the rate was 112 per minute, after he had a total of 72 grams of quinidine sulfate. Electrocardiogram at this time revealed a persistence of the ventricular tachycardia, with a rate of 132 per minute (Fig. 2F). The QRS complexes were markedly widened. The patient felt better. On September 18, the ventricular tachycardia gave way to a normal sinus rhythm with a rate of 84 per minute. By this time he had 150 grams of quinidine sulfate. Electrocardiogram (Fig. 2G) revealed marked inversion of the T waves in Leads II and III. The waves were large and broad. The QT interval was prolonged, and the ST junctions in these leads were depressed. The contours differed from the inverted T waves following the initial paroxysmal tachycardia (compare with Fig. 2B), and, in addition, T-4 remained upright.

Quinidine intake was reduced, but still administered. On September 23, electrocardiogram (Fig. 2H) showed a shallow T-1, with inverted T waves in Leads II and III, but not nearly so broadened as they were in the prior tracing. From then on, his recovery was uneventful. Quinidine was continued in dosages of 9 to 12 grains daily, and on October 18, his final electrocardiogram (Fig. 2I) revealed upright T waves in all leads.

**Comment.**—The preceding case is one of recurrent bouts of tachycardia over a period of fifteen years, only two of which were studied and which proved to be of the ventricular type.<sup>1</sup> It is believed that this patient had undoubted organic heart disease. The first paroxysm lasted four and a half days and terminated without the use of quinidine. This was followed by inversion of the T waves in Leads II, III, and IV F, with depression of the S-T junctions in the same leads. After a period of almost two months, the T waves became upright in Lead II, diphasic in Lead III. The second paroxysm lasted six days and was terminated after he had received 150 grains of quinidine sulfate as well as prostigmine, over a period of four days. Again the T waves progressed through a similar evolution, although differing in configuration and duration from those following the first attack, which had been uninfluenced by quinidine. T-4 did not become inverted following the second attack. After a period of one month, all T waves reverted to an upright position, and during this period quinidine had been continued in maintenance doses.

The question of myocardial infarction must be considered. However, it is believed that it did not take place because of the following reasons: There was no chest pain, and, other than the arrhythmia, there was nothing which suggested an infarct, clinically. Electrocardiographically, there were no reciprocal S-T deviations and no Q wave evolved. The S-T junctions were depressed, rather than elevated, in the leads showing T-wave inversion. The reversal of T waves occurred quite rapidly (one month following the second paroxysm), and the changes were very similar to those following the first paroxysm. Finally, the history of often repeated paroxysms of rapid heart action makes it appear unlikely that an occlusion with a myocardial infarct ushered in each of those attacks.

**Case 3.**—A. H. M., a 48-year-old white male, was admitted Oct. 5, 1942, because of genitourinary difficulty. He had had hematuria, associated with the passage of gravel, for eight years prior to admission. Three weeks before, he had a chill, and this recurred daily and was associated with a temperature rise. He was treated for malaria at home. Associated with the chill, he noticed a "red" urine, which continued almost up to the time of admission. A day or so before entrance, he had a pain in his right flank which radiated back to the right costovertebral region.

were sclerotic. There was no evidence of congestive failure. The remaining findings were not relevant. Carotid sinus pressure failed to alter the rate significantly. The electrocardiogram revealed a ventricular tachycardia (Fig. 2 A).

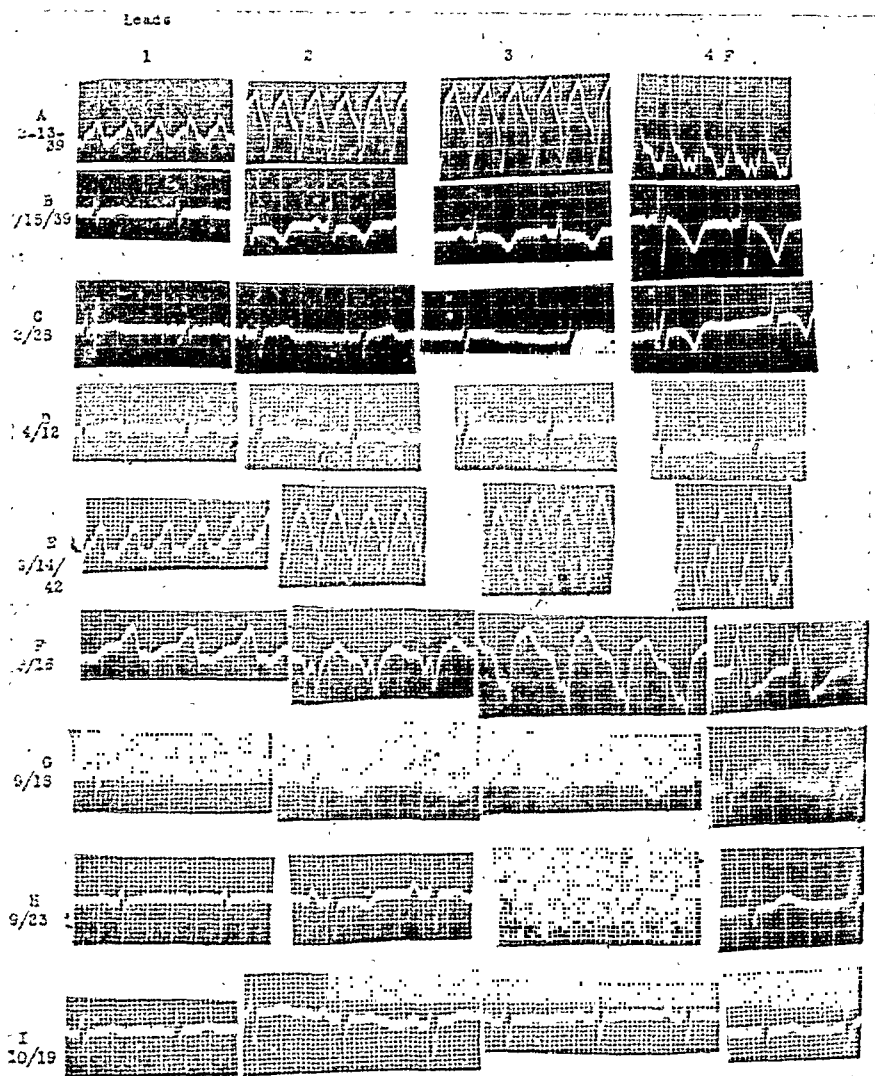


Fig. 2.

*Course.*—The patient remained afebrile throughout his hospital stay. No friction rub was audible. He was given small doses of digitalis, and the sinus rhythm became re-established on Feb. 15, 1939, the paroxysm lasting about four and a half days. Electrocardiogram (Fig. 2B), obtained Feb. 15, 1939, revealed marked inversion of the T waves in Leads II, III, and IV F, with depression of the S-T junction in Leads II, III, and possibly IV F. No quinidine was administered, but digitalis was continued in small daily doses, the patient probably having never been fully digitalized. The electrocardiogram of February 28 revealed a normal sinus rhythm, a diphasic plus-minus T-2 and T-3, and a deeply inverted T-4 (Fig. 2C), the rhythm continuing regular sinus. His course continued uneventful, and the last electrocardiogram during this admission (Fig. 2D) revealed an upright T-2, a very shallow, probably diphasic, plus-minus T-3, and a shallow, inverted T-4. The U waves were prominent in all leads. The patient was discharged as having attained maximum benefit, Apr. 16, 1939.

The sounds remained good. No friction rub appeared, and the blood pressure rose. The patient felt well. Quinidine therapy was then instituted, and on October 10, the electrocardiogram (Fig. 3 C) revealed an upright T-1, an isoelectric T-2, with a depressed S-T junction in both of these leads. T-4 inversion became more shallow and was giving way to diphasism. S-T-4 still remained depressed. On October 16, the tracing (Fig. 3 D) showed no essential changes in the limb lead with the exception of inversion of P-3, but T-4 was now upright, and there remained only minimal depression of the S-T junction in this lead. Quinidine had been administered in the same doses, after the second electrocardiogram was obtained, throughout his hospital stay. He refused ecologic workup and was discharged as asymptomatic soon thereafter. The cause for the eosinophilia was not established.

*Comment.*—The major T-wave changes occurred in Lead IV F, with lesser changes in Lead I, and the reversion to an upright position required but nine days following cessation of the ventricular tachycardia. There were no other electrocardiographic changes which might have unequivocally suggested an infarction. Moreover, there were no clinical findings to support such a contention. The S-T depression in Lead I remained that in Leads II and IV F disappeared. No Q wave appeared. The S-T deviations were not reciprocal and were not of the type associated with a known infarction pattern. Moreover, there were no serial changes to support the original contention of a left lateral wall infarct.

#### DISCUSSION

The case of supraventricular tachycardia failed to respond to the usual methods, including meclothyl in the dosage given. It persisted over eleven hours and terminated abruptly. T-wave inversion in Leads I and IV persisted for a period slightly less than two days. There was no evidence of organic heart disease. The mechanism of T-wave inversion following tachycardia is, in general, not known. Whether the patient had an "occult" coronary sclerosis which became manifest with a relative cardiac anoxia as a result of the tachycardia is conjectural. Whether "myocardial fatigue" alone can cause this inversion, in the presence of an intact, sufficient coronary circulation, has not definitely been proved, although it has been suggested by some.<sup>6, 8</sup> That the T-wave changes did not result from the quinidine is probable, in view of the relatively small dosage and the rapid return to an upright position, even though quinidine administration had been continued. She had, also, on various occasions, taken quinidine in similar doses, with no effect on her prior electrocardiograms.

A rather important point in this respect is the relatively infrequent association of paroxysmal auricular tachycardia with myocardial infarction. In a large series of acute coronary occlusions, Rosenbaum and Levine<sup>5</sup> did not observe one case, although other arrhythmias were not uncommon. Master and his co-workers<sup>7</sup> reported three cases in a group of 300 patients. It, accordingly, would appear, from a statistical standpoint at least, that T-wave inversion following supraventricular tachycardias is usually not due to an associated infarct, although only careful observation, both clinically and electrocardiographically, may be necessary before any definite diagnosis can be made, especially if the T-wave inversion persists for a matter of weeks. The important point to remember is that such inversion does occur and may persist for a period as long as two months, in the absence of organic heart disease, not to mention myocardial infarction.

That the duration of the attack bears no relationship to the length of T-wave inversion is suggested by comparing the time required for the reversion of the

T wave in Case I with the case of Geiger's.<sup>2</sup> Both had a paroxysmal tachycardia of supraventricular origin, of comparable duration, and yet Geiger's patient had persistent T-wave inversion for twenty-six days, as compared with less than two days for this case.

When ventricular tachycardias result in T-wave abnormalities, the diagnostic problem with reference to ruling out coronary occlusions is much more difficult. Following the first paroxysm in the second case, such a diagnosis was entertained, in view of the long persistence of the T-wave inversion, a period of almost two months. Following the second paroxysm, of six days' duration, T-wave inversion persisted for only slightly more than one month. This again suggests that there does not appear to be any relationship between the duration of inverted T waves and the length of the causative paroxysm.

The reasons for not entertaining quinidine as the cause of the T-wave inversion in this and in the third case have been discussed previously mainly because similar inversion existed following both paroxysms in the second case, even though no quinidine had been given during or following the first attack, and because following the second attack, the T waves returned to an upright position during the administration of quinidine. In the third case no quinidine was administered during the attack. In fact, in our experience with ventricular tachycardias treated with massive doses of quinidine, T-wave inversion was the exception rather than the rule, unless it was part of the electrocardiographic evolution of a "protracted myocardial insufficiency."

The importance of proper evaluation of these electrocardiographic changes, from a therapeutic and prognostic standpoint, cannot be overemphasized. To lean toward the side of conservatism, with respect to therapy, is, on the whole, commendable, but the avoidance of erroneous diagnoses carrying ominous prognoses should be strived for. It is believed that cardiac invalidism has been enforced upon many patients because of failure to appreciate the possibilities of the mechanisms described.

The probability of an associated infarction must be considered etiological in these T-wave abnormalities if Q waves appear, if progressive changes in reciprocal S-T deviations are evolved (in occlusions the S-T intervals are elevated in those leads where the T waves become inverted) and if other clinical factors suggesting an infarct coexist. These changes probably do not occur in "benign," even though persistent, T-wave inversion associated with and following paroxysmal tachycardia.

#### SUMMARY

1. One case of supraventricular tachycardia and two cases of ventricular tachycardia followed by T-wave inversion in multiple leads, persisting for a variable period of time and not associated with myocardial infarction, are presented.

2. The importance of correct evaluation of these changes is stressed.

3. The role that quinidine played is discussed. It does not appear to have been of etiological importance.

4. Certain differential electrocardiographic findings are discussed, and their importance in excluding myocardial infarction is stressed.

5. Persistent inversion of T waves following tachycardias is not necessarily of ominous prognostic import.

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Each subject was furnished with clean treated hose as needed and was instructed to wear a clean pair each day, reporting any unpleasant sensations. After each day's wearing, the hose were returned to the laboratory where they were laundered, mended, and reimpregnated. The soiled socks were soaked in tap water at 40° centigrade for 30 minutes, rinsed, soaked again in tap water at 70° centigrade for 30 minutes for the destruction of the fungi responsible for athlete's foot. They were then washed in soapsuds, rinsed, dried, and impregnated with the copper salts.

#### RESULTS

Table I shows the kind and concentration of copper salts used, a brief description of the skin condition at the beginning of the period of treatment, the duration of infection prior to treatment, the length of time the socks impregnated with copper salts were worn, and the skin condition at the close of the study.

The skin of 6 subjects appeared to be normal at the close of the period of observation. That of 11 others was improved. The skin condition of one (M. C.) became worse. It is believed that the condition of two of these (B. B. and C. M.) might have shown further improvement had the subjects been more conscientious in wearing the socks. Complaints of irritation were not made when the acetate was used, but one subject out of six objected to the odor. The acetate seemed to be more beneficial than the sulfate. Itching was usually relieved within a week. No harmful effect on the shoes was reported.

#### SUMMARY

Eighteen subjects with clinical athlete's foot wore cotton hose impregnated with copper sulfate or acetate for periods of 1½ to 10 months. The skin of six appeared normal, eleven showed improvement, and one was worse at the end of the experiment. The acetate seemed to be more beneficial than the sulfate.

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# THE EFFECT OF HEAT PRODUCED BY SHORT WAVE DIATHERMY ON THE ACTIVITY OF MUSCLE\*

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## INTRODUCTION

ALTHOUGH some form of heat has been employed since earliest times in the treatment of pain, injury, and weakness of skeletal muscle, the problem of its use is perplexing because there is incomplete scientific evidence as to what effect heat has on the intact muscle. The problem is further complicated by the variety of methods employed for applying heat and the resulting attempt to evaluate the efficiency of each. Since short wave diathermy is a form of heating widely used in the treatment of muscle, the experiment reported here was undertaken in an attempt to find out how its application affects the duration of the various phases of muscular contraction and to what degree each phase is affected.

An examination of the literature yields but little experimental data concerning the effects of the application of heat on the response of intact muscle.

Denkman<sup>1</sup> studied the effect of heat generated by the infra red lamp (radiation) on the response of the intact gastrocnemius muscle. Twenty-four subjects were used. Her conclusions were that after twenty minutes of radiation there was a shortening of the latent and contraction periods of muscular activity. No definite conclusions were drawn from her data on relaxation time.

Tuttle and Williams<sup>2</sup> studied the effect of autocondensation and high frequency currents (conversion) on the tonus of skeletal muscle. The extent of the knee jerks of nine subjects, before and after heating, was compared. It was found that the autocondensation current when applied in sufficient quantities to cause perspiration reduces the extent of the reflex. The increase in temperature evidently causes a relaxation of the muscles and thus a decrease in tonus.

Other experiments pertinent to the subject of the effects of short wave diathermy on muscle are reviewed by Bierman.<sup>3</sup>

This experiment was conducted on twenty normal individuals, sixteen women and four men, between the ages of twenty and forty years. The group was selected at random. Each individual's muscular response was recorded before rest and after twenty minutes of rest. On the following day at the same hour his muscular responses were recorded before heat and after twenty minutes of application of heat with short wave diathermy. The data on the effects of rest were collected so as to be sure that any effects appearing in the heat records could not be attributed to rest. The contraction of the intact gastrocnemius muscle was obtained by applying an electric stimulus to the motor point.

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*The Stimulating Unit.*—The source of the stimuli was the laboratory type of inductorium. One lead from the secondary winding of the inductorium was terminated in a copper electrode  $\frac{3}{4}$  of an inch in diameter, which was covered with electrode jelly. This electrode was strapped over the medial motor point of the gastrocnemius muscle. The other pole of the secondary winding was terminated in a metal electrode with a surface area of 6 by 6 inches. This electrode covered with wet Canton flannel was placed on the outer part of the thigh. The primary winding of the inductorium was placed in series with a hand switch, two dry cells, and a signal magnet. Submaximal break induction shocks were used as stimuli.

*The Recording Device.*—The data were recorded on the smoked paper of an extension kymograph.

The subjects were placed prone on a table. A stout string was attached to the sole of the subject's shoe by a thumbtack. The string extended under the table and to a recording stylus suspended from a rubber band. Uniform tension was maintained on the string at all times by means of the rubber band. The time of the application of the stimulus was marked on the record by a signal magnet placed in the primary circuit. An electrically driven tuning fork vibrating at the rate of 100 double vibrations per second was placed between the signal magnet and the stylus attached to the subject's foot.

After the electrodes were applied, the tension on the muscle adjusted, and the recording device ready, the drum was spun, during which time the stimulus was delivered and the response recorded. Since all the recording styli were on the same ordinate, the speed of the kymograph was immaterial except that it was fast enough so that the time could be read to the nearest quarter of a double vibration. The greatest number of complete contractions which it was possible to record on one drum was recorded each time. The number of recordings found in the data were those which could be read with confidence as to validity.

*The Heating Unit.*—The portable, short wave diathermy machine used was an "Intratherm" Model P125, manufactured by the Therapeutic Oscillator Corporation, West Des Moines, Iowa.

*The Record.*—Records were made as stated in the section on the recording device. The first records were those of the response of the muscle before rest. The subject was allowed to rest for twenty minutes and then records were made of the response of the muscle. The subject returned the next day at the same hour and a record was made of the response of the muscle before heating. Then the electrodes from the inductorium were removed and a cuff condenser electrode was tied around the leg just below the knee. The other cuff condenser electrode was tied around the leg just above the ankle. The subjects were free to move the leg throughout the heating period. They were told to inform the investigator if the leg felt too hot all over, too hot in any one spot, or if there was a sensation of muscle cramping. The desired sensation was one of comfortable warmth. The current was turned on and left on for twenty minutes. The intensity of the current was regulated according to the subject's heat tolerance. After the heating period, the heating electrodes were removed, the electrodes from the inductorium replaced, and a record of the response of the muscle was recorded.



*Reading the Record.*—The technique of recording required that the response of the gastrocnemius muscle be represented as a downward movement on the smoked drum. However, for purposes of computation, the records were turned over and read from right to left. They are represented in this manner in Fig. 1. The top line is made by the recording stylus attached to the foot, the lower line by the signal magnet, and the middle one by the 100 double vibrations tuning fork. The procedure for calculation consisted of dropping an ordinate through the break in the signal magnet line, then one through the point at which the recording stylus left the base line. In order to establish a

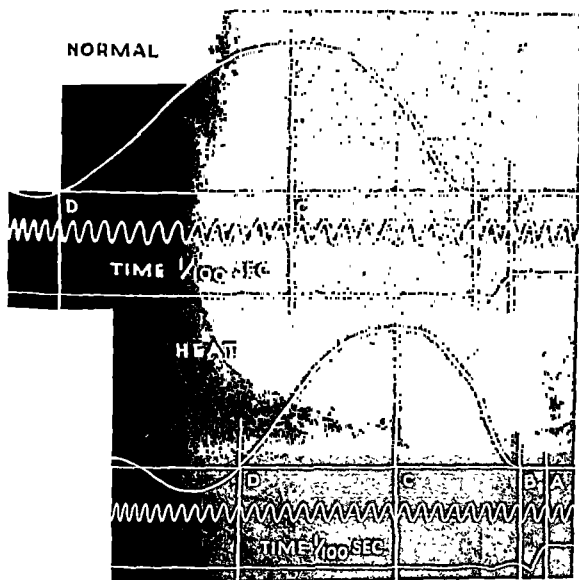


Fig. 1.—Record of the effect of heat produced by short wave diathermy on the activity of muscle. The upper figure shows the normal muscular response. The lower figure shows the muscular response after heat. Reading from right to left.

#### Normal

A-B	Latent period	0.0175 sec.
B-C	Contraction period	0.0875 sec.
C-D	Relaxation period	0.1250 sec.

#### Heat

A-B	Latent period	0.0175 sec.
B-C	Contraction period	0.0775 sec.
C-D	Relaxation period	0.0950 sec.

base line, the recording stylus line, before contraction occurred, was continued through the record. An ordinate was dropped through the middle of the flat-test part of the top of the contraction curve, and finally an ordinate was dropped through the time line from the point where the recording stylus returned to the base line. It is evident that the latent time is the time elapsing between the

stimulus and the leaving of the base line by the recording stylus. The contraction time began at the place where the stylus left the base line and ended at the middle of the flattest part of the curve. The relaxation period began at the end of the contraction time and ended at the point where the stylus returned to the base line.

The duration of each period of the form curve was determined in seconds by reading the number of tuning fork vibrations between ordinates.

*The Data.*—A summary of the data relative to the effect of rest on the activity of the intact muscle is presented in Table I. The data show that no significant changes in the activity of the muscle were caused by a period of rest.

TABLE I  
THE EFFECTS OF REST ON MUSCULAR ACTIVITY

PERIODS OF MUSCULAR ACTIVITY	GROUP MEANS		DIFFERENCES	S.E. DIFF.	t*	PER CENT CHANGE
	BEFORE	AFTER				
	sec.	sec.	sec.			
Latent	.0196	.0191	-.0005	.0006	0.8	2.6
Contraction	.1020	.1012	-.0008	.0014	0.6	0.8
Relaxation	.2464	.2546	.0082	.0046	1.8	3.3
Total time	.3680	.3700	.0020	.0067	0.3	0.5

\*Differences are not significant at the 5 per cent level of confidence unless t is 2.1 or more.

Table II shows the effect of heat produced by short wave diathermy on the various phases of muscle activity. Each period of the form curve was significantly shorter after the muscle was exposed to heat. The greatest shortening effect occurred in the relaxation period. This conforms to the generally accepted idea that whatever alters the duration of the periods of the form curve produces the most pronounced effect on the period of relaxation.

TABLE II  
THE EFFECTS OF HEAT ON MUSCULAR ACTIVITY

PERIODS OF MUSCULAR ACTIVITY	GROUP MEANS		DIFFERENCES	S.E. DIFF.	t*	PER CENT
	BEFORE	AFTER				
	sec.	sec.	sec.			
Latent	.0196	.0158	-.0037	.0009	4.1	19.0
Contraction	.1009	.0893	-.0116	.0033	3.5	11.5
Relaxation	.2543	.1976	-.0567	.0077	7.4	22.3
Total time	.3746	.3027	-.0719	.0073	9.8	19.2

\*Differences are significant at the 1 per cent level of confidence when t = 2.9 or more.

It is of interest to compare the effects of cooling obtained by Tuttle<sup>4</sup> with those of heat reported here. An ice pack applied to the gastrocnemius muscle for 20 minutes prolonged its activity twice as much as the application of heat for 20 minutes shortened it. Obviously, this is due to the fact that the temperature of the muscle was lowered by the ice considerably more than the temperature was raised by short wave diathermy.

#### CONCLUSIONS

The effect of the application of short wave diathermy to the gastrocnemius muscle was investigated. The data show that all periods of the form curve of contraction were significantly shortened by the application of heat.

Although all periods were significantly shortened, the period of relaxation was changed most.

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# THE EFFECT OF ARTIFICIALLY INDUCED FEVER ON ANAPHYLACTIC SHOCK IN ACTIVELY SENSITIZED GUINEA PIGS\*

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IN COLLABORATION WITH

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DEKRUIF and Simpson<sup>1</sup> have recently pointed out a mechanism of desensitization for the favorable effect of fever in conditions thought to be of an allergic nature. These include conditions of the rheumatic state, intractable asthma and urticaria, and treatment of dermatitis following injections of arsenicals in patients being treated for syphilis. Gernez and Eloire<sup>2</sup> studied a small series of serum-sensitive guinea pigs and found that they were desensitized at the height of fever and that the high fever afforded protection. Hyperpyrexia was induced by a temperature rise of six to seven degrees Fahrenheit in 15 to 20 minutes. Mirsky and Wassermann in collaboration with Kravitz<sup>3</sup> desensitized serum-sensitive guinea pigs by placing them in a bath of 45°C. for 15 minutes until their rectal temperatures were 109°C. and then gave them a fatal dose of serum at the height of fever; the animals were desensitized. In both of the experiments reported here, the temperatures were raised very rapidly. Since clinical experience with the technique by which the fever is administered varies considerably, the individual's reaction during the treatment,<sup>4</sup> we first tested the method as entirely safe for normal animals and which would produce febrile temperatures in human subjects. The results of the protection by hyperpyrexia in serum-sensitive guinea pigs are reported here.

## METHODS

Groups of 10 to 20 albino guinea pigs of an average weight of 500 grams were given by subcutaneous injection of 1.0 c.c. of a 1:100 dilution of serum. These animals were sufficiently sensitive to 23 to 136 c.c. of serum and had been given. Both sexes were used, the age ranging from 12 to 25 weeks and the weight 575 to 915 grams. They were chosen because they were found to be less excitable than other strains of guinea pigs in fever and in shock. The serum was injected into the back of the neck of the febrile and control guinea pigs. Immediate

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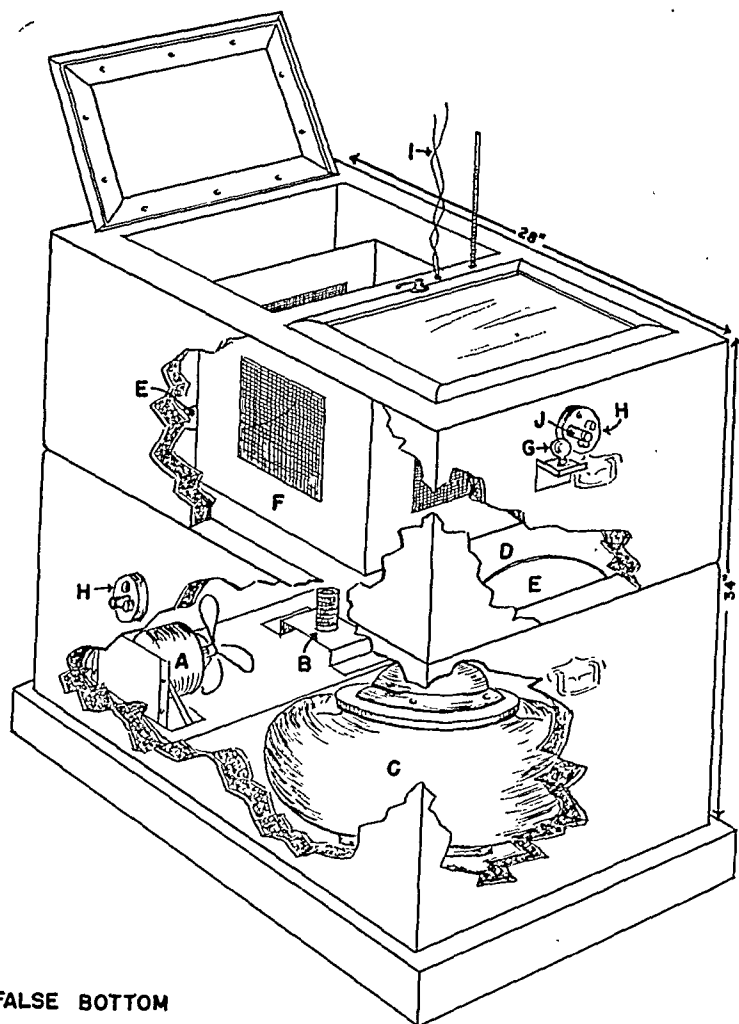
animals, the amount of serum fatal for 80 to 100 per cent of these animals was established. Generally five to eight animals were required for this titration. This amount of serum was then used as the assaulting dose for each febrile animal and its unfebrile control. The assaulting dose of serum, therefore, varied from experiment to experiment, and if two groups of animals were used the dose sometimes varied within an experiment.

For induction of fever, hyperpyrexia induced by short waves was found to be unsatisfactory, since it was difficult to follow the rise in the animal's temperature because the thermometer had to be removed from the rectum during radiation. A large humidified cabinet related in principle to the hypertherm developed at the Kettering Institute for Medical Research for the production of hyperpyrexia in human beings was then tried. The animal was fastened to a modified Latapie board, the thermocouple inserted into the rectum, and the animal pushed into the cabinet. The temperature of the cabinet was then increased at a rate which did not raise the rectal temperature more than  $1.0^{\circ}$  F. during any five-minute period. This method was found to be satisfactory, although struggling of some animals occasionally led to an accelerated rise in their body temperature.

The method found simplest and most satisfactory was to place the guinea pig in a small air-conditioned cabinet, illustrated in Fig. 1.\* A humidifier maintained the humidity of the cabinet at a point approaching saturation. The copper-constantan thermocouple, made with No. 30 B. and S. gauge wire, was prepared as described by Robinson.<sup>7</sup> Either rectal or subcutaneous temperature measurements were made. For rectal temperature measurements the couple was inserted into the rectum through a 2 mm. glass tube to a depth of four to six cm. The tube was then withdrawn by sliding it up the wire, care being taken to keep the couple in place in the rectum. The wires were fastened to the guinea pig with adhesive tape to prevent the couple from slipping out. The animal was allowed freedom of movement in a small cage within the cabinet. For subcutaneous temperature measurements a  $1\frac{1}{2}$ -inch 19 gauge needle was pushed through the skin to a depth of about three cm. It was removed immediately, and the couple was inserted in its place to a depth of two to three cm. The couple was then fastened to the animal with adhesive tape, and the animal was placed in the cage within the cabinet.

After the guinea pig was placed in the cabinet, the glass-windowed doors, permitting observation of the animal, were closed, and the heat and humidifier were turned on. The cabinet temperature was raised to from  $96$  to  $102^{\circ}$  F. The dry heat regulated by the rheostat was adjusted to produce a slow increase, or a constant temperature, as found necessary to give the desired degree of hyperpyrexia. For maintaining the animal at the desired temperature, the cabinet temperature was usually lowered several degrees by removal of the rubber stoppers from the ventilators. The humidifier maintained the humidity close to 100 per cent of saturation throughout the fever session. Trials with normal animals preceded each experiment in which a different temperature was studied. This was done to determine the best method for regulating the cabinet

\*The humidifier and heating device was installed by Mr. Edwin Sittler, Liebel-Flarsheim Co., Cincinnati, Ohio, formerly associated with the Kettering Institute for Medical Research.



### KEY

- A - FAN
- B - HEATER
- C - HUMIDIFIER
- D - FALSE BOTTOM
- E - AIR VENTS IN FALSE BOTTOM
- F - ANIMAL CAGE
- G - 15 W. BULB FOR I.V. INJECTION
- H - VENTILATORS ADJUSTABLE WITH RUBBER STOPPERS
- I - THERMOCOUPLE LEADS
- J - 1 1/4" DIAMETER BRASS TUBE

Fig. 1.—Diagrammatic drawing of the small animal cabinet used for producing hyperpyrexia in guinea pigs.

temperature to obtain satisfactory induction and maintenance of fever. Too rapid a rise in the rectal temperature caused by too high a cabinet temperature and sudden changes in cabinet temperatures were avoided. Cabinet and rectal temperatures were recorded at five-minute intervals. It was not possible to predict what cabinet temperature would raise an animal's temperature to a certain height, since individual guinea pigs varied somewhat in their response to temperature and humidity. Typical fever curves obtained by the use of this method are given in Fig. 2.

After the desired degree of hyperpyrexia had been attained and maintained for the intervals specified in Tables I, II, and III, an assistant qu

removed the animal, placed its nose and mouth in a short brass tube  $1\frac{1}{4}$  in. in diameter, leading into the cabinet. This precaution was taken to prevent the animal from breathing the cooler air of the room during injection. The injection was made into an ear vein, and the animal was returned to the cabinet immediately. A 15 watt bulb fastened to the cabinet just below the guinea pig's ear facilitated injection. Removal of the animal from the cabinet, injection, and return to the cabinet usually required less than three minutes. The injection

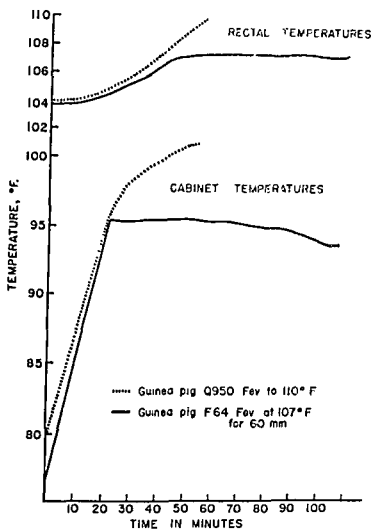


Fig. 2.—Rectal and cabinet temperatures during fever treatment of guinea pigs.

was made with a 0.5 or 1.0 c.c. syringe and a short leveled 27 gauge needle. Five minutes after the injection was made, the cabinet doors were opened and defervescence was allowed to take place spontaneously. Thirty to sixty minutes later the animal was transferred to its cage. Those animals that recovered lost weight for from 48 to 72 hours, but usually attained their prefever weight within a week.

The arbitrary method of recording the severity of anaphylaxis was as follows:

**Severe:** Severe respiratory distress in which the animal coughed, scratch its nose, hunched up, and gasped for breath. Cyanosis was marked, and the animal was prostrated. This was followed by death or recovery.

**Moderate:** Respiratory distress of shorter duration in which the animal coughed and scratched its nose but was not prostrated. Cyanosis was slight or absent.

TABLE I  
THE EFFECT OF ELEVATIONS OF TEMPERATURE ON ANAPHYLACTIC SHOCK IN GUINEA PIGS

EXPERIMENT	FEVERED ANIMALS										UNFEVERED CONTROL ANIMALS						APPROXIMATE AGE IN WEEKS
	FEVER IN DEGREES FAHRENHEIT	NO. OF ANIMALS	DIED < 1 HR.	DIED 1-24 HR.	RECOVERED	SEVERITY OF SHOCK			NO. OF ANIMALS	DIED < 1 HR.	DIED 1-24 HR.	RECOVERED	SEVERITY OF SHOCK				
						SE-VERE	MODERATE	SLIGHT					SE-VERE	MODERATE	SLIGHT		
																NONE	
1	108°	5	3	2	2	3	2		3	5	1	1	5			12	
2	108°	5	4	1	1	5			5	4			5			22	
3	109°	5		3	2	2	1		5	5			1			13	
4	110°	5			3		3		5	4			5			14	
5	110°	5		2*	3	1	2		5	3			2			21	
6	110°	5		1	4				5	4			1			16	
7	110° and inj. with saline	5		5	5				5	4			1	1		20	
8	109° to 111.2° Goldman method	7		5	2		3	1	7	6			1		7	25	

\*One of these animals died 2½ days after administration of horse serum.



Slight: The fur was ruffled; slight respiratory symptoms were evident as indicated by scratching the nose or sneezing. The animal was usually excited. Cyanosis and coughing were not observed.

#### EXPERIMENTAL

Five sensitized guinea pigs were artificially fevered to rectal temperatures of  $108^{\circ}$  F., and each was injected with a dose of horse serum lethal for 80 to 100 per cent of the control unfevered animals. In this experiment the animals were restrained as previously described. As shown in Table I, exp. 1, three animals which developed severe symptoms of anaphylaxis died within four minutes, while two developed moderate symptoms of anaphylaxis and recovered. The five control animals died in less than five minutes. When five animals were fevered to  $108^{\circ}$  F. but were not restrained, the same number of deaths occurred in the fevered and the control groups (Table I, exp. 2). These experiments show that little protection is afforded by a fever temperature of  $108^{\circ}$  F. when a fatal dose of serum is given as soon as this temperature is attained.

Five sensitized guinea pigs were then fevered to rectal temperatures of  $109^{\circ}$  F. and immediately injected with a dose of horse serum lethal for the control unfevered animals. Table I, exp. 3, shows that in this group in which the animals were restrained during induction of fever, none of the animals died of acute anaphylactic shock, although three died 3 to 24 hours later and two recovered. These results indicate that this temperature affords some protection against acute anaphylactic shock. In this and later experiments no correlation was found between the severity of shock and death 1 to 24 hours later.

Three groups of sensitized guinea pigs were then fevered to  $110^{\circ}$  F., and as soon as this temperature was attained a lethal dose of horse serum was injected. In these and subsequent experiments the animals were not restrained. In experiment 4 subcutaneous temperature measurements were taken, while in experiments 5 and 6 rectal temperature measurements were made. In these three experiments in which a total of 15 animals were used, only one animal showed severe symptoms; five showed moderate, seven slight, and two no symptoms of shock, indicating that a certain degree of protection is also afforded at this temperature. There seemed to be no significant difference in protection, whether the temperature was taken by the subcutaneous or by the rectal route. In another experiment subcutaneous and rectal temperatures were compared in four guinea pigs during induction of fever. The results which are not given in detail indicated that after a rectal temperature of  $106^{\circ}$  F. was attained, the subcutaneous temperature in three animals was from  $1.5$  to  $2.0^{\circ}$  lower than the rectal temperature. In the other animal the subcutaneous and rectal temperatures were equal throughout the induction period.

An attempt was then made to determine whether deaths from 1 to 24 hours following administration of serum were due to fever. Five sensitized guinea pigs were fevered to  $110^{\circ}$  F. and at the height of fever were injected with saline. As shown in Table I, exp. 7, none of these animals died, indicating that the late deaths were probably due not to fever but to anaphylactic shock or anaphylactic shock superimposed on fever.

TABLE II

THE EFFECT OF ELEVATIONS OF TEMPERATURE WHEN THE SHOCK DOSE WAS ADMINISTERED AT VARYING INTERVALS AFTER TEMPERATURE HAD BEEN ATTAINED

EXPERIMENT	FEVERED ANIMALS										UNFEVERED CONTROL ANIMALS										APPROXIMATE AGE IN WEEKS
	FEVER			NO. OF ANIMALS	DIED			RECOVERED	SEVERITY OF SHOCK			NO. OF ANIMALS	DIED			RECOVERED	SEVERITY OF SHOCK				
	DEGREE FAHRENHEIT	DURATION IN MINUTES	UTES		< 1 HR.	1-24 HR.	SEVERE		MODERATE	SLIGHT	NONE		< 1 HR.	1-24 HR.	SEVERE		MODERATE	SLIGHT	NONE		
1	109°	30	5		3	2	1				5	5	5	5		5	5	5	5	17	
2	108°	30	5		1	4		1			5	5	5	5		5	5	5	5	17	
3	108°	30	5		2	3	2	2			5	5	5	5		1	5	5	5	16	
4	107°	60	5		3	2	3	3			5	5	5	5		1	5	5	5	16	

TABLE III

THE EFFECT OF THE AGE OF ANIMALS ON THE RESPONSE TO AN ASSAULTING DOSE OF SERUM ADMINISTERED AFTER A FEVER TEMPERATURE OF 108° F. HAD BEEN MAINTAINED FOR 30 MINUTES

EXPERIMENT	FEVERED ANIMALS										UNFEVERED CONTROL ANIMALS										APPROXIMATE AGE IN WEEKS
	FEVER			NO. OF ANIMALS	DIED		SEVERITY OF SHOCK			RECOVERED	SEVERITY OF SHOCK			RECOVERED	SEVERITY OF SHOCK						
	DEGREES FAHRENHEIT	DURATION IN MINUTES			< 1 HR.	1-24 HR.	RECOVERED	SEVERE	MODERATE		SLIGHT	NO. OF ANIMALS	< 1 HR.		1-24 HR.	SEVERE	MODERATE	SLIGHT			
1	108°	30	6	2	1	3	4	2						1	6			7			
2	108°	30	8			8	1	1	4	2				1	8			112			

The method of Mirsky, Wassermann, and Goldman<sup>3</sup> was then tried, in which method the body temperature was elevated very rapidly. The humidified cabinet was heated to from 111 to 112° F., and the animals were placed in it for 15 minutes. At this time their rectal temperatures varied from 109 to 111° F. A lethal dose of horse serum was then given. As shown in Table I, exp. 8, none of these guinea pigs died of acute anaphylactic shock, although five of the seven animals died within 24 hours after receiving the assaulting dose of serum.

A group of five guinea pigs was then subjected to a fever temperature of 109° F. and maintained for 30 minutes to determine whether better protection was afforded by maintaining them at this temperature for 30 minutes than by merely bringing them to this temperature and injecting the specific antigen immediately. The data given in Table II, exp. 1, indicate that maintenance of this temperature for 30 minutes affords slightly better protection than if the injection of serum is made as soon as 109° F. is reached.

To ascertain whether sensitized guinea pigs were protected by longer treatment at temperatures at which protection was not afforded by merely bringing them to these temperatures before injection, two groups of guinea pigs were fevered at 108° F. for 30 minutes and another group at 107° F. for 60 minutes. In these series shown in Table II, exp. 2, 3, and 4, none of the animals died of acute anaphylactic shock, although one to three of each group died within 1 to 24 hours after receiving a dose of horse serum lethal for 80 to 100 per cent of the controls. It appears that if the animals are held for 30 minutes at 108° F. or for 60 minutes at 107° F., the same protective effect is obtained as if they are brought to temperatures of 109 or 110° F. and the specific antigen injected immediately.

In induction of fever in human subjects the fluid balance is maintained by administration of large quantities of 0.6 per cent saline and other fluids, but unfortunately most guinea pigs refuse water or food during fever treatment. Five guinea pigs were therefore injected with 4.0 c.c. of normal guinea pig serum intraperitoneally immediately before being fevered at a temperature of 107° F. for 60 minutes. In this experiment two animals died within 10 minutes, one died 1 to 24 hours after administration of horse serum, and two recovered, indicating that injection of normal guinea pig serum previous to fever treatment did not eliminate shock or late deaths.

Since the age of the guinea pigs in the former experiments varied considerably, an experiment was made in which desensitization by artificially induced fever was compared in young and in old guinea pigs. These animals were fevered to rectal temperatures of 108° F., and this temperature was maintained for 30 minutes, after which a lethal dose of horse serum was injected. The age of the young group, composed of four males and two females, was seven weeks, and the age of the old animals, composed of six females and two males, was approximately 112 weeks. The results given in Table III show that young animals are much more difficult to protect than old ones. Four of the young animals showed severe symptoms and two showed moderate anaphylactic symptoms, while in the older group, one showed severe, one moderate, and four slight symptoms of shock, while two were completely protected. Two of the young

animals died within four minutes and one within 24 hours, whereas none of the older animals succumbed. The lethal dose of serum for the young animals was from 0.075 to 0.1 c.c., and for the older group it was from 0.35 to 0.75 c.c. It appears, therefore, that older animals do not become very sensitive and are easily desensitized, while young animals become very sensitive and are also more difficult to desensitize.

Most of the fevered and control animals that died within 1 to 24 hours were autopsied. The gross findings revealed marked congestion of the peritoneal wall, intestines, and stomach. In most cases the liver, spleen, kidneys, and lungs did not appear abnormal. A study of histologic sections of these tissues by one of us (H.E.C.) indicated that there was no essential difference between the findings in fevered and unfevered animals, except for a larger amount of lymphoid tissue in the lungs of all of the fevered animals dying within 12 hours. In general, significant histologic changes were confined to the lungs, kidney, liver, and spleen. In animals dead within 1 to 24 hours, varying degrees of extravasation of blood into the pulmonary alveoli and into the parenchyma of the medullary portion of the kidney, the liver, and the spleen were seen. Occasionally there were cuff hemorrhages in the cortex of the brain and some hemorrhage in glomerular spaces of the kidney. Evidences of smooth muscle spasm in the form of constricted pulmonary arterioles or bronchi were not a prominent or consistent finding. As the time interval between fever and death lengthened, the picture changed somewhat. Extensive hemorrhage in lungs, kidney, and spleen became the most prominent feature, and blood pigment was seen throughout these organs. Degenerative changes of both fatty and granular type were seen in the liver.

#### DISCUSSION

Kendell and Simpson<sup>2</sup> have shown that luetic patients sensitive to arsenical drugs can tolerate large doses of these chemicals during hyperpyrexia at temperatures 7.4 to 7.9° F. above the normal temperature (106.0 to 106.5° F.). Since the mean rectal temperature of a group of 57 of our normal guinea pigs at an environmental temperature of 65° F. was 102.8° F., the guinea pig temperature corresponding to the temperature used in treatment of human patients would be approximately 110° F. At this temperature we have obtained protection against acute anaphylactic deaths. While 110° F. in the guinea pig corresponds to the temperature at which human patients are treated, it did not appear that our animals could tolerate this temperature for any length of time because some of them were weak and listless. They did not, however, drag their hind feet as if paralyzed as described by Goldman,<sup>3</sup> although this was occasionally observed after the assaulting dose of horse serum had been given.

Deaths 1 to 24 hours following administration of serum in fevered guinea pigs have been encountered. These deaths did not occur when sensitized guinea pigs were fevered and injected only with saline. Furthermore, late deaths have not occurred in those animals used for trial runs to determine the best method for regulating the cabinet temperature to obtain satisfactory induction and maintenance of fever, nor in those animals which were fevered but for some reason were not injected with serum. Neither do late deaths occur very frequently when unfevered sensitized guinea pigs are injected intravenously with

horse serum, as shown by the protocols of the control animals given in Tables I, II, and III. Since these late deaths are not due to fever and do not occur very frequently in unfevered sensitized guinea pigs injected with serum, they are probably the result of anaphylactic shock superimposed on fever. It is suggested that these late deaths constitute another type of anaphylactic effect.

In our experiments three groups of animals of different ages were febrile at 108° F., and this temperature was maintained for 30 minutes before the assaulting dose of horse serum was administered. One group was seven weeks old, the other approximately 16 weeks (Table II, exp. 3), and the oldest group was 112 weeks of age. The 7-week-old animals obtained the least protection by this treatment; the 112-week-old animals, the most protection; while the 16-week-old animals were better protected than the youngest group of animals, but not as well protected as the oldest group.

#### SUMMARY

Sensitivity to horse serum in mature serum-sensitive guinea pigs is suppressed by artificially induced fever temperatures of 109 or 110° F. when the serum is injected as soon as these temperatures are attained.

Fever temperatures of 108° F. do not suppress anaphylactic shock if the animals are injected with serum as soon as they reach this temperature, but if this temperature is maintained for 30 minutes a certain degree of protection is afforded.

Serum-sensitive guinea pigs, 112 weeks old, are more readily protected than young guinea pigs, 7 weeks old, when subjected to a fever temperature of 108° F. for 30 minutes.

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# THE CLUMPING OF ERYTHROCYTES IN HAYEM'S DILUTING FLUID: A GRAVE PROGNOSTIC SIGN\*

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THE clumping of red cells, occasionally seen in Hayem's diluting fluid when performing an erythrocyte count, is a phenomenon which has been known for a number of years and has been observed in various pathologic conditions. This phenomenon is not uncommon and in our opinion has not received due consideration as a possible grave prognostic sign.

In his book, Gradwohl<sup>1</sup> reported, purely as a matter of record rather than as a diagnostic measure, three independent cases of clinical and pathologic Hodgkin's disease in which a peculiar condition of the red cells was noted. Distinct clumping of the cells occurred when the blood was diluted with Hayem's solution, making it difficult to perform an accurate erythrocyte count. The blood did not display any clumping in these cases, however, when diluted with a 2.5 per cent citrate of soda solution.

Kracke and Hoffman<sup>2</sup> in reporting a case state that "the erythrocyte count was impossible with Hayem's diluting fluid because of marked clumping of the cells. A slight amount was present even when normal saline was used." Other similar reports are included with our observations in Table I.

Since no references in the literature were found attaching any prognostic importance to this manifestation, we considered it worth while to report some preliminary observations.

## DESCRIPTION

In the clumping of the erythrocytes in Hayem's solution in the red blood cell pipette, the clumps, grossly, are small but vary in number and size, the larger ones sedimenting rapidly. (Fig. 1) They occur soon after dilution and appear to be permanent. Microscopically they appear as irregular entangled masses of red blood cells; some contain only a few cells, others a great many. As noted earlier, this definitely interferes with an accurate cell count, to a greater or lesser degree depending on the intensity of the reaction, which varies in different cases.

## OCCURRENCE AND THEORY

The condition has been found to occur in a great number of entirely different diseases; however, we believe it to be based on the same fundamental blood change. In our study the cause was not determined, and although in some cases it might have been due to autoagglutination, in most instances it was probably an exaggeration of rouleau formation. This formation of irregular

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clumping sometimes simulates true agglutination, but is more properly called "pseudoagglutination." Belk<sup>3</sup> points out that "differences of opinion exist as to whether the pathologic cold agglutination and rouleau-forming substance are the same." He shows evidence that there is a difference. No doubt some cases of clumping of erythrocytes in Hayem's diluting fluid are brought about by autoagglutination, but most cases of autoagglutination have not shown this display.

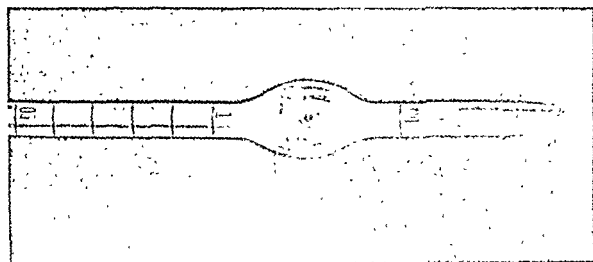


Fig 1

It has been found to occur in all ages of both sexes and in various races (white, Negro, Indian.) Attempts to correlate this phenomenon with changes

TABLE I A  
DATA SHOWING CLUMPING OF ERYTHROCYTES IN HAYEM'S  
SOLUTION IN INFECTIONS

DISEASE	OUTCOME		
	DEAD	LIVING	UNKNOWN
1. Pneumonia	5	2 (17 yrs. of age)	1
2. Bronchopneumonia	2		
3. Lung abscess	1		
4. Staphylococcus	1		
5. Streptococcus	2		
6. Subacute bact.	2		
7. Puerperal sepsis	2		
8. Abscess of kidney (Polycystic kidney and liver disease)	1		
9. Pyelonephritis—Bilateral (renal calculi)	2		
10. Pyonephrosis	1		
11. Pyelitis (also diabetes)	1		
12. Acute purulent otitis media (Staphylococcus septicemia)		2 (Age 4)	
13. Ulcerative colitis	1		
14. Upper respiratory infection (child)		1 (Age 4)	
15. Peritonitis (following appendicitis)	1		
16. Brain abscess			1
17. Cervical adenitis (child)	1		
18. Salpingitis			1
19. Meningitis (typo?)	1		
20. Nephritis	1		
21. Rheumatoid arthritis (transfusions)	1		
22. Cholecystitis (transfusions)	1		
23. Chronic hemolytic anemia <sup>1</sup>	1		
24. Atypical pneumonia (acrocyanosis following) <sup>4</sup>		1	

in the blood protein, nonprotein nitrogen, sugar, white cell and differential counts, erythrocyte count, platelet count, blood groups, pathologic urine findings, fever, blood transfusion, etc., have failed. It may be associated with a high sedimentation rate and increased plasma protein, but it may also be seen with normal or even low protein levels.

TABLE I B  
DATA SHOWING CLUMPING OF ERYTHROCYTES IN HAYEM'S  
SOLUTION IN NEOPLASMS AND ALLIED DISEASES

DISEASE	OUTCOME		
	DEAD	LIVING	UNKNOWN
1. Adenocarcinoma of stomach	1		
2. Scirrhus carcinoma of stomach	1		
3. Cancer of breast—carcinomatosis (1 had secondary thrombopenic purpura)	2		
4. Cancer of pancreas	1		
5. Cancer of kidney—carcinomatosis	1		
6. Cancer of throat	1		
7. Cancer ampulla of Vater	1		
8. Brain tumor (type?)	1		
9. Lymphatic leucemia (chronic) (1 had lobar pneumonia)	2		
10. Chronic leucemia (type?) <sup>4</sup>	1		
11. Myelogenous leucemia	1		
Myeloid leucopenic	1		
Leucopenic with secondary thrombopenic purpura	1		
12. Hodgkin's disease	3		
13. Multiple myeloma <sup>5-9</sup>	More than 5 (many)		

TABLE I C  
DATA SHOWING CLUMPING OF ERYTHROCYTES IN HAYEM'S  
SOLUTION IN MIXED, METABOLIC, NONINFECTIOUS DISEASES

DISEASE	OUTCOME		
	DEAD	LIVING	UNKNOWN
1. Cardiac (chronic) failure	2		
2. Several transfusions (reason unknown)	1		
3. Burns		1 (Aged 16 years)	
4. Diabetes	1		
With cholecystitis and coronary occlusion	1		
Cardiorenal vascular disease	1		
5. Pernicious anemia	1		
6. Anaphylactoid purpura (terminal subacute glomerulonephritis, hepato- titis, and bronchopneumonia)	1		
7. Essential hypertension	2		
8. Chronic nephritis (acute pericarditis, terminal)	1		
9. Raynaud's disease <sup>10</sup>		1	
10. Benzene poisoning (thrombopenic purpura)	1		

Tables I and II summarize the number and types of cases in which this reaction has been observed by us. In view of the number and variety of different diseases reported, it was not considered necessary to give complete case reports.



TABLE II  
DATA OF TOTAL NUMBER OF CASES

		DIAGNOSED	UNDIAGNOSED
Total cases	105	72	33
Known dead	76	60	16
Known recovered	13	7	6
Outcome unknown	16	5	11

#### PROGNOSTIC SIGNIFICANCES

When this phenomenon occurs in a patient and is particularly intense, the usual outcome is death. Unfortunately it may make its appearance days, weeks, or even months before the patient's time of death. Although seen in all age groups, it is definitely more important and less variable in older age groups. The majority of cases in which the patient was known to have recovered were children. It is worthy of note that the reaction was reversible in cases where death did not occur. In general, slight or weak reactions were observed in cases that recovered, while strong reactions practically always indicated death as the final outcome.

#### SUMMARY

A phenomenon, namely the clumping of erythrocytes in Hayem's diluting fluid when performing a red cell count, has been described, and pathologic states in which it occurs have been tabulated. The possible importance of this phenomenon as a grave prognostic sign is offered.

#### ADDENDUM

A recent paper by Shone and Passmore<sup>12</sup> reveals that autohemagglutination is found almost constantly in atypical pneumonia, being more pronounced in the severe cases. This apparently minimizes the graveness of the phenomenon, but stimulates fresh interest for the study of the fundamental manifestation.

The authors wish to thank Major Carmelo De Angelis, Washington, D. C., and Dr. Percival Mae Lachlan, Morgantown, W. Va., for their valuable help in preparing this paper.

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# REACTIONS IN THE BLOOD AND ORGANS OF DOGS ON INTRAVENOUS INJECTION OF A SOLUTION OF HEMOGLOBIN\*

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THE presence of free hemoglobin in the blood plasma may be the result of poisonings (potassium chlorate, hydrogen arsenide, phenylhydrazine, naphthol, toluylene diamine, saponin, photodynamic fluorescent hydrocarbons and dyes [sulfonamides, acridine dyes]), blackwater fever, paroxysmal and cold hemoglobinemia, and resorption of large internal hemorrhages. In recent years hemoglobinemia has assumed an increased significance, because it may follow upon the transfusion of incompatible blood or of hemolyzed blood. Attempts have also been made to use solutions of hemoglobin freed from cellular stromata as a substitute for whole blood and plasma in the treatment of shock (Amberson;<sup>1</sup> Cannan;<sup>2</sup> O'Shaughnessy, Mansell, and Slome<sup>3</sup>). Such solutions can carry oxygen and maintain colloidal osmotic pressure for a period of up to 36 hours, after which they become ineffective because of the transformation of hemoglobin into methemoglobin and the escape of the injected hemoglobin from the blood with subsequent elimination through the kidneys (Lichty, Havill, and Whipple;<sup>4</sup> Klingmüller;<sup>5</sup> LaCoste, Aubertin, and Castagnou;<sup>6</sup> Yuile, Steinmann, Hahn, and Clark;<sup>7</sup> Gilligan, Altschule, and Katersky;<sup>8</sup> Hesse and Filatov<sup>9</sup>).

While it is a well-established fact that the intravascular hemolysis or the injection of hemolyzed blood may give rise to severe reactions, particularly anuria, caused by the blockage of the tubular lumina by hemoglobinous pigments and cellular detritus, such complications are less frequent or entirely lacking when solutions of pure hemoglobin are introduced (DeGowin, Osterhagen, and Andersch;<sup>10</sup> DeGowin, Warner, and Randall;<sup>11</sup> O'Shaughnessy, Mansell, and Slome<sup>3</sup>). In view of the practical and scientific significance of this aspect and as an additional contribution to the study on the effects of parenterally injected macromolecular substances (Hueper<sup>12</sup>), solutions of hemoglobin were injected into the veins of dogs.

## EXPERIMENTAL PROCEDURE

A 2 per cent solution of hemoglobin (hemoglobin, pure, scales (Pfanstiehl)) in normal saline was injected into the jugular veins of three dogs (8.2 kg. to 10 kg.). One dog received 7 c.c. and two dogs 20 c.c. each of this solution. Blood studies were made 5, 15, 30, and 60 minutes, 2, 4, and 24 hours later to ascertain the acute hematologic reactions. After an interval of three days, increasing amounts of hemoglobin solution were introduced daily five times per week and the blood was examined in weekly intervals. The individual dose was gradually

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raised to 70 c.c. within 5 weeks. The dogs were sacrificed by the intravenous injection of formalin solution after 6 weeks of treatment, during which 28 injections were given. A total of 1,390 c.c. of hemoglobin solution or 28 Gm. of hemoglobin were introduced into each dog during the experimental period.

The injections were usually well tolerated. Occasionally there occurred a short attack of dyspnea, rapid pulse, and, rarely, vomiting. After the first week of injections the urine showed a trace of albumin. During the six weeks of treatment the three dogs lost from 0.9 to 1.8 kg. of body weight.

#### ACUTE HEMATIC REACTIONS AFTER THE INTRAVENOUS INJECTION OF A SINGLE DOSE OF HEMOGLOBIN SOLUTION

Immediately after the intravenous injection of the hemoglobin solution the hemoglobin content of the blood was increased by 1 Gm. per cent to 1.8 Gm. per cent for a period of up to two hours. This increase was caused in part by the hemoglobin injected, as the plasma was distinctly hemolytic during the first 4 hours and slightly hemolyzed after 24 hours, in part it was the result of a transitory increase in the number of circulating erythrocytes (increases up to 1.8 million). The number of leucocytes, on the other hand, dropped sharply 5 to 15 minutes after the injection to values as low as 600 from an original level of 7,700 to 11,400. This leucopenia was accompanied by a relative lymphocytosis of moderate to marked degree. The original level was re-established after 30 minutes in the dog which received 7 c.c., but there was still a distinct lowering of the number of leucocytes in the other two dogs which were injected with 20 c.c. four hours after the injection. All three dogs revealed in the 24-hour test a considerable leucocytosis (22,000 to 33,200) associated with an increase of the neutrophilic cells and the appearance of an excessive number of immature leucocytes.

The volume of packed blood cells was slightly reduced at the 24-hour period, at which time there was also noted a moderately accelerated sedimentation of the erythrocytes (13 to 14 mm. Wintrobe-Landsberg hematocrit tubes). The coagulation time remained within normal limits in two dogs but showed 60 minutes after the injection an excessive lengthening (up to 2 hours) in the dog which had the most severe hemoclastic crisis. Normal values were present in all three dogs at the 24-hour period. The plasma viscosity, determined with the viscosimeter of Hess, remained within normal limits.

#### HEMATIC REACTIONS AFTER REPEATED INTRAVENOUS INJECTIONS OF HEMOGLOBIN SOLUTION

The repeated intravenous injection of hemoglobin solution caused a slow but progressive drop in the amount of hemoglobin, in the number of erythrocytes, and in the hematocrit values. The hemoglobin was reduced by approximately one-third of its original value toward the end of the 6-week period, while the number of erythrocytes had decreased by approximately the same value or more (30 to 50 per cent). The number of leucocytes fluctuated between 12,000 and 68,000 and was usually between 20,000 and 30,000. The sedimentation rate was considerably and consistently accelerated (30 to 55 mm.). The coagulation time and plasma viscosity remained within normal limits.

## PATHOLOGY

The organs of the three dogs were grossly normal. Upon histologic examination the brain of one dog showed a moderately dense lymphocytic infiltration of the meninges and relatively dense perivascular lymphocytic accumulations around many of the intracerebral vessels. In some areas of the cortex there was a proliferation of capillaries and an increase of glia cells. The brains of the other two dogs were normal. The outer media of the aortas of two dogs showed in the region of the arch a crescent-shaped, large, hyaline, fibrohyaline, or mucohyaline area which contained an increased number of vasa vasorum with thickened walls and a moderate lymphoid cell infiltration. The inner media of the ascending aorta revealed a moderate to marked mucoid infiltration. The descending aorta was normal as were the larger aortic branches (carotid artery, femoral artery). The livers contained groups of Kupffer cells heavily loaded with a dark brown amorphous pigment. These storage phenomena were moderately frequent in the liver of one dog and scanty in those of the other two dogs. The liver cells of the three dogs had an almost unstained, coarsely flaky cytoplasm. The reticulum cells of the splenic pulp contained large amounts of brown pigment in one dog, scanty amounts in a second dog, and were almost free from pigment in the third dog. This animal, however, showed numerous megakaryocytes scattered throughout the pulp. The kidneys of two dogs were normal, while those of the third dog revealed moderate vacuolar changes in the tubular epithelium. None contained any brown pigment in the tubular epithelium or tubular lumina. The arteriolar walls in the testis of the one male dog were thickened and showed small, tongue-like endothelial processes. All other organs (hypophysis, thyroid, heart, lung, pancreas, stomach, intestine, adrenals, prostate, bladder) were normal. The bone marrow of the sternum consisted of a dense immature myeloid tissue.

## COMMENT

The recorded observations show that the intravenous introduction of a relatively small amount of a comparatively dilute hemoglobin solution elicits a typical hemoclastic crisis, such as noted previously after the administration of other macromolecular substances (Hueper<sup>12</sup>). This hematic reaction is characterized by a primary transitory leucopenia with relative lymphocytosis, a secondary leucocytosis with absolute neutrophilia, increased sedimentation rate, and lengthened coagulation time. The moderate loss of body weight, the progressive and appreciable reduction in the number of erythrocytes and of the amount of hemoglobin, the considerable acceleration of the erythrocytic sedimentation, and the, at times, excessive leucocytosis represent manifestations following the repeated and prolonged intravenous administration of macromolecular material. It may be added that Gilligan, Altschule, and Katersky<sup>8</sup> under similar experimental conditions in man, and Yuile, Steinmann, Hahn, and Clark<sup>7</sup> found in dogs a lowering of the excretion threshold of the kidneys for hemoglobin.

It is remarkable that the histologic study of the various organs of the three dogs produced relatively little anatomic evidence pointing to a toxic action of the injected hemoglobin. It may be possible that the coarsely flaked appearance

of the liver cells indicates the presence of an extraneous proteinic matter interfering with the proper function of these cells. The hematogenous pigmentation of the Kupffer cells and of the splenic reticulum cells, on the other hand, are not sufficiently pronounced to cause any serious functional damage to these organs. The kidneys were practically without any significant lesions and showed a complete absence of any pigmentary deposits in the tubular epithelium and tubular lumina.

It is uncertain whether or not the meningo-encephalitis existing in one dog, the medial degenerations in the aorta of all three dogs, and the arteriolar thickenings in the testes of the one male dog have any causal connection with the treatment given or are coincidental findings. It may be mentioned in this connection, however, that Gilligan, Altschule, and Katersky<sup>8</sup> reported the occurrence of cramps in patients injected with hemoglobin solution and that Hesse and Filatov<sup>9</sup> observed marked spasms of the renal arterioles after the injection of hemoglobin solutions into animals, while von Berency<sup>13</sup> noted in a case of hemoglobinemia due to naphthol poisoning a hemoglobinous imbibition of the aortic wall.

#### CONCLUSIONS

1. The intravenous injection of hemoglobin dissolved in normal saline elicits a hemoclastic crisis followed by a leucocytosis within 24 hours.
2. Two of the three dogs used showed after the first injection an untoward reaction (dyspnea, rapid pulse, vomiting, bowel movement, urination, restlessness). None of the subsequent 27 injections was followed by such a response.
3. Repeated daily injections of hemoglobin solutions cause loss of weight, a considerable decrease in the number of erythrocytes and in the amount of hemoglobin, appreciable and, at times, excessive leucocytosis, and markedly accelerated erythrocytic sedimentation.
4. There was no formation of hemoglobinous casts in the tubular lumina and only a mild to moderate deposition of a hemoglobinous pigment in the liver and spleen.
5. The acute and chronic hematic and organic reactions follow in many respects the pattern set by observations made following the intravenous introduction of other macromolecular substances.

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# CLINICAL CHEMISTRY

## ASSAY OF RENIN SUBSTRATE BY LOW TEMPERATURE INCUBATION WITH RENIN\*

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THE formation of angiotonin by the reaction between renin and renin substrate has been established to be enzymatic in nature (Plentl and Page<sup>1</sup>) (Leloir, et al.<sup>11</sup>). When tissue extracts are used as sources of renin and renin substrate, destruction of the angiotonin formed by this reaction occurs through the agency of the enzyme hypertensinase (Fasciolo, Leloir, and Munoz<sup>2</sup>) [angiotonase] (Helmer, Kohlstaedt, and Page<sup>3</sup>).

Renin and renin substrate are most conveniently assayed by determination of the angiotonin produced by their interaction. For the determination of renin, excess substrate is used, and the angiotonin produced is measured after constant incubation time (Leloir, Munoz, Braun-Menendez, and Fasciolo<sup>4</sup>). Renin substrate is determined by incubating it with excess renin and measuring the angiotonin produced after the reaction has gone to completion.<sup>4</sup>

In such methods, an accurate assay depends on the conversion of renin substrate to angiotonin *without consequent destruction of the angiotonin by hypertensinase*. This may be accomplished by destroying the hypertensinase present in tissue extracts. Two methods for inducing this destruction have been described (Dell'oro and Braun-Menendez;<sup>5</sup> and Braun-Menendez<sup>6</sup>), but unfortunately one of the methods results in partial destruction of renin substrate and is therefore useless when renin substrate determinations are desired, and the other has failed, in our hands, to destroy hypertensinase activity completely.

Page and Helmer<sup>7</sup> have demonstrated that the reaction between renin and renin substrate is decelerated but not stopped by decreasing the incubation temperature to 0° C. Furthermore, they observed no destruction of angiotonin in the time of their run (1½ hours). We have repeated and extended these observations, and have demonstrated that a decrease in temperature inhibits the destruction of angiotonin by hypertensinase far more than it does its formation by the renin-renin substrate reaction. At 0° C the former reaction is entirely arrested, while the latter continues to completion, though at a diminished rate. We have made use of this phenomenon in the development of a technique for the quantitative assay of renin activator.

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## MATERIALS

## 1. Renin:

Renin of sufficient purity for the present experiments and for ordinary pharmacologic investigations can be prepared in 2 to 12 hours (depending on the size of the batch) by the following technique:

Hog kidney (meat market kidneys are satisfactory) is ground through a meat grinder and agitated with five times its weight of 2 per cent NaCl. The mixture is run through a colloid mill, or blended with ice in a Waring blender until homogenous. HCl is added with stirring to pH 4.1. The precipitate is removed by supercentrifugation or by filtration through Hyflo Supercel. An equal volume of saturated ammonium sulfate solution is added slowly to the filtrate, and the precipitate which is formed is filtered off on Hyflo Supercel. The filter cake is broken up, agitated with a volume of water equal to the original weight of kidney, and dialyzed in Visking tubing for 1 to 12 hours. The shorter times may be used if a rocking dialyzer and thin tubing are employed. The filtercake remaining in the solution is removed by filtration and the filtrate is saved. This solution, which is free of depressor activity and contains from 2 to 4 renin units (Swingle, Taylor, Collings, and Hays<sup>8</sup>) per c.c., is kept on ice or lyophilized. Lyophilized preparations contain approximately one gram of solids per pound of kidney.

This method has been applied to the preparation of renin from dog, rat, and hog kidneys with excellent results, but horse kidneys, for unknown reasons, fail to yield any pressor activity.

## 2. Renin substrate:

Renin substrate may be prepared from beef serum as described previously (Sapirstein, Southard, and Ogden<sup>9</sup>), or it can be assayed directly in serum or plasma.

## 3. Angiotonin:

A standard angiotonin for control of the pressor assays is prepared from renin and renin substrate by incubating 1 gram of lyophilized renin with 1 liter of renin substrate (equivalent to 5 liters of serum) for 2 hours at 0° C. The reaction is stopped by the addition of sufficient 50 per cent trichloroacetic acid to yield a final concentration of 5 per cent. The precipitate is removed by filtration, and the filtrate is boiled over a free flame for 30 minutes to remove the trichloroacetic acid (Peters and Van Slyke<sup>10</sup>). The solution is concentrated until 1 c.c. represents 10 c.c. of serum. One c.c. of this solution, which is stored on ice at pH 1.5 and neutralized immediately before use, contains 4 pressor units (Braun-Menendez, et al.<sup>11</sup>) and 0.03 mg. of non-ammonia N.

## INCUBATION AND ASSAY METHODS

One gram of the standard lyophilized kidney powder is dissolved in 100 c.c. of 1 per cent NaCl. One c.c. of this solution is incubated with 10 c.c. of serum, plasma, or concentrated renin substrate for varying periods, and at different temperatures. At 0° the incubation is carried out for 0, 30, 60, 90, 120, and 1,000 minutes; at 25° for 7, 10, 15, 22, 30, and 45 minutes; and at 37° for 0, 3, 6, 9, 12, 15, and 18 minutes. The reaction is stopped by plunging the tubes into a boiling water bath and stirring violently while they are heating. Addition of



an equal volume of N/20 acetic acid during the heating facilitates the precipitation and results in a clearer filtrate. The precipitate is removed by filtration or centrifugation and the supernatant is assayed for pressor activity.

#### ESTIMATION OF PRESSOR ACTIVITY

Cats nembutalized with sodium pentobarbital are used. The blood pressure is recorded from the carotid artery. After administration of the usual anesthetic dose of sodium pentobarbital (35 mg. per kilo), the blood pressure is ordinarily found to be elevated and unstable and assays are inaccurate. This may be remedied by the slow administration of more nembutal via the femoral vein until the blood pressure drops to from 60 to 80 mm. Hg. At this level the blood pressure remains stable and the animal is quite responsive to angiotonin. Chlorotone anesthesia, which produces a similar stable low pressure, has been found unsuitable for angiotonin assay, as the sensitivity is decreased threefold to tenfold.

The angiotonin content of the unknown solution is determined by comparison of its pressor activity with that of standard angiotonin. When it has not been possible to obtain identical responses from both standard and unknown, we have calculated the strength of the unknown angiotonin from the equation recommended by Braun-Menendez.<sup>11</sup>

$$\frac{(\text{Pressure rise unknown})^2}{(\text{Pressure rise standard})^2} = \frac{\text{Units of unknown}^{11}}{\text{Units of standard}}$$

#### FINDINGS

The curves of Fig. 1A represent the angiotonin produced (plotted in units) at incubation temperatures of (a) 0°, (b) 25°, and (c) 37° C. In Fig. 1B, these three curves have been collated on an arbitrary, sliding time scale, all times being represented as the percentage of average value of the time required for maximum formation of angiotonin at each temperature.

TABLE I  
AMMONIUM SULFATE FRACTIONATION OF RENIN SUBSTRATE IN BEEF SERUM

FRACTION	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> CONCENTRATION	RENIN SUBSTRATE (UNITS/50 C.C. SERUM)
I	0-25	0
II	25-30	1
III	30-33	2
IV	33-36	8
V	36-40	5
VI	40-50	1

These results confirm those of Schales.<sup>12</sup>

#### DISCUSSION

It may be seen by inspection of Curve 1B that the ratio  $\frac{\text{rate of destruction of angiotonin}}{\text{rate of formation of angiotonin}}$  decreases with temperature and that it assumes a value of 0 at 0° C.; i.e., hypertensinase activity is completely inhibited at this temperature. We have observed this elimination of hypertensinase activity in partially hemolyzed blood, and even upon incubating crude saline extracts of kidney with

whole blood. Since these contain large amounts of hypertensinase, it may be assumed that incubation at  $0^{\circ}$  C. completely inhibits the hypertensinase activity of tissue extracts.

The elimination of hypertensinase activity by  $0^{\circ}$  incubation, while renin activity remains, makes possible the easy, accurate assay of renin substrate by a procedure which prevents the destruction of formed angiotonin, and at the same time does not involve the partial destruction of renin substrate by measures designed to effect the chemical destruction of hypertensinase.

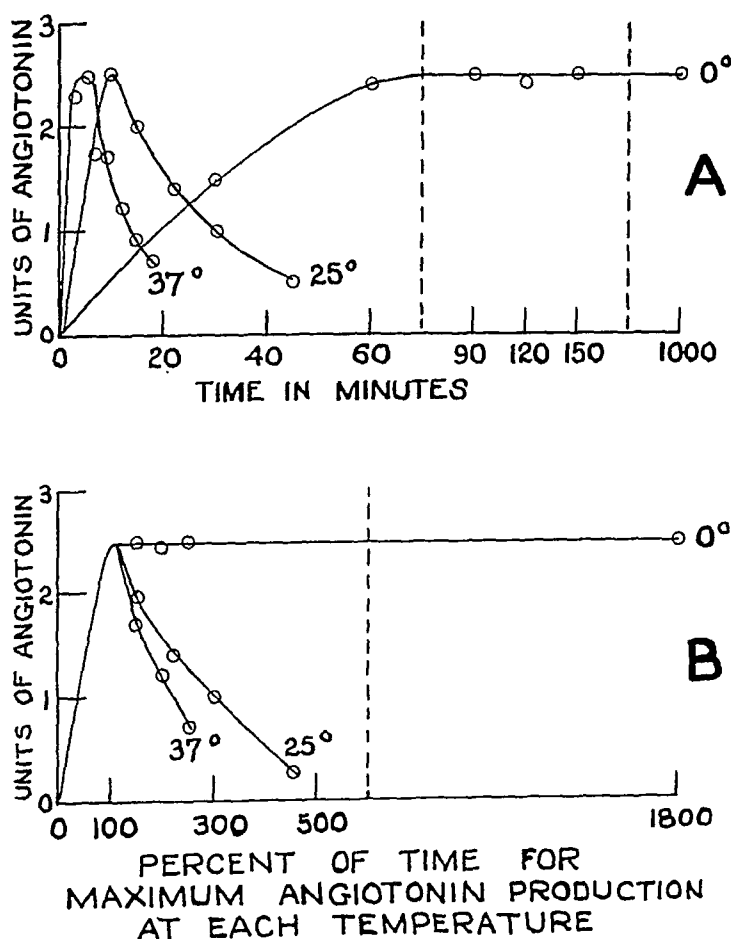


Fig. 1.—Effect of temperature on the formation of angiotonin by the action of renin on renin substrate.

A, Angiotonin production at  $37^{\circ}$ ,  $25^{\circ}$ , and  $0^{\circ}$  C. plotted against time in minutes.

B, Angiotonin production at  $37^{\circ}$ ,  $25^{\circ}$  and  $0^{\circ}$  C. plotted against time as per cent of time required for maximum production of angiotonin at each temperature.

#### APPLICATIONS

1. Assay methods: Renin substrate can be determined in whole blood, serum, plasma, or substrate concentrates without preliminary treatment by the following technique:

One part of lyophilized renin (see materials) is dissolved in 100 c.c. 1 per cent NaCl. One c.c. of this solution is incubated with 10 c.c. of the solution to

be assayed for two hours at 0° C. A control incubation of zero time is set up simultaneously. The reaction is stopped in a boiling water bath, according to the technique described above. Dilution with acetic acid is desirable in the case of whole plasma or serum but unnecessary when substrate concentrates are being assayed. The supernatant solution is assayed for pressor activity as previously described.

We define one unit of renin substrate as that amount of renin substrate which gives rise to one unit of angiotonin when the incubation is performed as described.

Using this assay technique, we have determined the substrate concentrations of various fractions of beef serum obtained by fractional precipitation with ammonium sulfate. Our results are expressed in Table I.

2. Preparation of angiotonin: Large batches of angiotonin can be prepared by the 0° incubation method as described above (materials). No precautions are necessary to avoid hemolysis, nor is the usual rigid time control, which is advisable in order to stop the reaction when maximum formation of angiotonin has occurred, necessary.

#### SUMMARY

1. A method for the quantitative assay of renin substrate based on the inhibition of hypertensinase activity at 0° C is described.

2. The bulk of the renin substrate of ox serum is precipitable between 0.33 and 0.40 saturation with ammonium sulfate.

3. Methods for the preparation of renin and angiotonin are described.

We wish to express our thanks to Mr. A. Robinson for preparation of the figure.

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# LABORATORY METHODS

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## GENERAL

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### INDIRECT SPHYGMOMANOMETRY

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#### A PHYSICAL AND PHYSIOLOGIC ANALYSIS AND A NEW PROCEDURE FOR THE ESTIMATION OF BLOOD PRESSURE

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MANY sphygmomanometric devices and methods have been devised for indirect blood pressure estimation. All procedures thus far described in the literature cannot be considered totally free from error under clinical conditions. The object of this investigation, therefore, is to evaluate the sources of error in the indirect sphygmomanometric methods and to suggest procedures for eliminating or reducing the magnitude of these errors.

To proceed with any degree of success, we found it necessary to devise an apparatus which could register graphically and simultaneously all of the physiologic phenomena which must be evaluated when estimating blood pressure by the palpatory, oscillatory, and auscultatory methods. Such a device, obviously, was intended as a means for the elimination of the human element with its subjective differences in the appreciation of the physiologic signs and to make possible a quantitative comparison of the associated phenomena.

#### REVIEW OF LITERATURE

In 1733, Stephen Hales<sup>1</sup> performed an experiment in which he estimated the blood pressure in a horse. Hales inserted a cannula into the femoral artery and allowed the blood to rise in a vertical tube; from the height of the blood column, Hales calculated the pressure. In 1828, Poiseuille<sup>2</sup> substituted a mercury manometer for the Hales' tube, and in 1847 Ludwig<sup>3</sup> devised a direct recording manometer. Faivre,<sup>4</sup> Albert,<sup>5</sup> Müller and Blauel,<sup>6</sup> Dehan, Dubus, and Heitz,<sup>7</sup> continued the work.

In recent years, many refinements have been added to the Hales' apparatus. Some of the better known investigators who have made worth-while contributions to the art are Galatà,<sup>8</sup> Poillet, Dodel and Boucomont,<sup>9</sup> van Bogaert, Beerens, Lequime and Samain,<sup>10</sup> Giroux,<sup>12</sup> Dameshek and Loman,<sup>13</sup> Hamilton, Brewer, and Brotman,<sup>14</sup> and Macleod and Cohn.<sup>15</sup>

It is generally agreed that the direct communication method between artery and registration device is the most accurate means for measuring blood pressure. Possible errors in the direct method due to nonabsolute transmission of pres-

sure from artery to instrument, spastic contraction of the artery in which the cannula has been introduced, psychic factors, the difficulty at times of accurately ascertaining the exact zero pressure level, and a few other such factors may exist. Although the direct method is invaluable in the physiologic laboratory, the procedure of inserting a cannula into the artery of a patient is a decided limitation from a clinical standpoint.

Riva-Rocci<sup>16</sup> in 1896 introduced an indirect method for blood pressure estimation based upon the preliminary findings of Béhier,<sup>17</sup> Foster,<sup>18</sup> Landois,<sup>19</sup> Philadelphien,<sup>20</sup> von Basch,<sup>21</sup> and Potain.<sup>22</sup> The Riva-Rocci method utilized a controlled pneumatic cuff for compressing the arm (brachial artery) and the palpation of the radial artery. The first palpable pulse wave which appeared during a gradual decompression of the cuff was taken as a sign that the level of systolic pressure had been reached.

The original palpatory method of Riva-Rocci is in use today as a check on the auscultatory systolic pressure. The accuracy of the method is limited by the sensitivity of the fingers in detecting the first small waves of the pulse, which may result in an underestimation of the systolic pressure. The estimation of the diastolic pressure by the Riva-Rocci method is an extremely uncertain procedure.

In order to improve upon the Riva-Rocci palpatory method, Bing,<sup>23</sup> Pal,<sup>24</sup> Vaquez,<sup>25</sup> and others devised visual oscillatory indicators. These devices utilized a second cuff as a substitute for the fingers, which cuff transmitted the arterial pulsations into a device which produced a magnifying effect with resulting easier perception than was possible by palpation. Some years later a double cuff was used by Groedel and Miller<sup>26</sup> for the registration of the pulse.

The Riva-Rocci palpatory method and the oscillatory indicators, plus the investigations of Marey<sup>27</sup> and von Recklinghausen,<sup>28</sup> paved the way for the first practical oscillatory type sphygmomanometer by Pachon.<sup>29</sup> The Pachon apparatus immediately received wide usage and is in use to this day. It is composed of an aneroid manometer which indicates the cuff pressure and of a constantly balanced manometric capsule which indicates the magnitude of the arterial oscillations.

In using the instrument, the cuff is applied to the arm in the conventional manner, and its pressure is raised to a point above systolic. The cuff pressure is indicated on the aneroid manometer. Attention is then focused upon the indicator controlled by the balanced manometric capsule, and it is observed for oscillations. This procedure is repeated as the cuff pressure is brought down to below diastolic pressure, usually in steps of 5 to 10 mm. of mercury.

As the cuff pressure is gradually lowered (in steps) from above systolic, a series of oscillations of small amplitude appear (supramaximal oscillations). This series of oscillations has been attributed to the hydraulic ram action of the pulsating proximal stump of the artery against the edge of the cuff. The supramaximal oscillations are then followed by a marked and abrupt increase in the oscillatory amplitude (the level of systolic pressure); the oscillations continue to increase progressively in amplitude to a maximum, then gradually decrease, and then abruptly decrease followed by some small oscillations (infra-minimal oscillations).

The diastolic pressure was first thought to coincide with the maximal oscillation, as the arterial wall should then oscillate freely between two equivalent pressures (Marey<sup>27</sup>). As a result of the investigations of Macwilliam, and Melvin,<sup>30</sup> whose data were accepted by Gallavardin,<sup>31</sup> the diastolic level was placed much lower (where a marked change in amplitude occurs). Pachon and Fabre,<sup>32</sup> and Gley and Gomez<sup>33</sup> also confirmed the necessity of the change and accepted the data.

Gallavardin<sup>34</sup> later modified the Pachon oscillogram and the cuff, and concluded that the supramaximal oscillations must be due to the hydraulic ram action. Fabre<sup>35</sup> attributed the supramaximal oscillations to the elastic reaction of the artery; Gley and Gomez<sup>33</sup> attributed the phenomenon to the infundibulum formed by the upper part of the artery, and Minerbi and Merighi<sup>36</sup> to the pulsations of the *arteria brachialis profunda*. For more precise oscillographic investigations, recording oscillograms were devised by Plesch<sup>37</sup> (tonoscillograph) and Boullite<sup>38</sup> (arterial oscillograph).

The investigations of Strasburger<sup>39</sup> and Ehret<sup>40</sup> were continued by Korotkow.<sup>41, 42</sup> Korotkow, in 1905, suggested that blood pressure may be estimated by auscultation. The Korotkow method utilizes a Riva-Rocci apparatus and a stethoscope. Instead of palpating the artery, as is done by the Riva-Rocci palpation method, a stethoscope is applied to the brachial artery below the cuff. Five distinct phases may be heard and differentiated, as the cuff pressure is slowly decreased from above systolic pressure to below diastolic. The five phases occur in the following order: Sounds similar to the first cardiac sound; a similar series of sounds, plus a hissing murmur; the vanishing of the murmur with the sound remaining; a sudden muffling of the sound; the disappearance of the sound.

Since the introduction of the Korotkow auscultatory method, many differences of opinion have been expressed as to the true location of the diastolic pressure. At first, the level where the loudest sound occurred was considered the diastolic level. Later, the idea prevailed that the last sound should be taken as the diastolic level (Macwilliam and Melvin;<sup>30</sup> Erlanger<sup>43</sup>). At the present time, the general belief, as expressed by the Anglo-American Committee,<sup>44</sup> is that the level of systolic pressure is the beginning of the first sound phase or where the first sound regularly appears; the diastolic pressure where the sounds suddenly become dull and muffled. If there is a difference between that point and the level at which the sounds completely disappear, the American Committee recommends that the latter reading should be regarded also as the diastolic pressure. The British Committee believes that except in aortic regurgitation it is nearly always possible to decide the point at which the abrupt change comes, and that this is the only reading that should be recorded.

Indirect methods for blood pressure estimation, as previously mentioned, are subject to several possible sources of error. The instrument itself may introduce variable degrees of error, but a more serious source of error is the utilization of the human senses for quantitative measurement. Cook,<sup>45</sup> and more recently, Wright, Schneider, and Ungerleider<sup>46</sup> have demonstrated that marked discrepancies occur in the taking and interpretation of blood pressure by competent physicians.

Since Marey's investigations in 1881, subjective differences in the evaluation of the blood pressure have been appreciated. As a result graphic registration methods have been devised by Béhier,<sup>17</sup> Foster,<sup>18</sup> Landois,<sup>19</sup> Philadelphien,<sup>20</sup> Jaquet,<sup>47</sup> Gibson,<sup>48</sup> Erlanger,<sup>49</sup> Uskoff,<sup>50</sup> Silbermann,<sup>51</sup> Brugsch,<sup>52</sup> Münzer,<sup>53</sup> Fleischer,<sup>54</sup> and others. All of these devices operated on the sphygmographic principle. The instruments of Boullitte<sup>54</sup> and Plesch,<sup>57</sup> which have been mentioned, should also be included in this category.

More recently, Gomez and Langevin<sup>55</sup> utilized a piezo-electric quartz crystal and electronic amplifier to register the pulse. The characteristic advantages inherent in the piezo-electric crystal<sup>56, 57</sup> for this type of application were lost, because the pulse was transmitted to the crystal through a contact button, thus reverting back to the Potain method. This is, the arterial pulsation is registered through the uncertain transmission of a contacting button with the result that all the errors present in the old Potain method are still present and possibly even magnified.

Von Recklinghausen<sup>58</sup> observed that graphic registrations of the pulse during a deflation of the cuff from above systolic pressure to below diastolic show small changes in the shape of the pulse waves which indicate the lateral and end systolic pressure, and the lateral and end diastolic pressure. Bazett and Laplace<sup>11</sup> confirmed von Recklinghausen's observations.

Von Recklinghausen<sup>58</sup> in 1901 suggested that the width of the cuff may severely affect the accuracy of the blood pressure reading. He showed that a cuff which is too narrow gave too high a blood pressure reading. The correctness of von Recklinghausen's observation was confirmed by Gumprecht's<sup>59</sup> experiments on the cadaver with artificial circulation and by Müller and Blauel.<sup>6</sup> Janeway,<sup>60</sup> and Ragan and Bordley<sup>61</sup> on living human subjects. Ragan and Bordley show experimentally that "in some obese subjects the substitution of a wide (20 cm.) blood pressure cuff for the standard (13 cm.) cuff afforded more accurate auscultatory measurements of both systolic and diastolic pressure. In most subjects, however, the wide cuff yielded measurements which were too low."

#### DESCRIPTION OF APPARATUS

In Fig. 1A may be seen a schematic diagram of an apparatus which is capable of registering simultaneously all of the physiologic phenomena that must be evaluated when estimating blood pressure by the auscultatory, palpatory, and oscillatory methods.

The apparatus consists of a double chamber pneumatic cuff; the outer covering of the twin chambers is of the usual nondistensible material. The upper or pressure chamber is of the usual dimensions (13 × 25 cm.); the lower or registration chamber dimensions are 3 × 25 cm. The pressure and registration chamber dimensions may be varied to suit any particular application or may be placed in separate nondistensible bags; a single bag, however, simplifies the application procedure.

The pressure chamber of the cuff connects to a manometer of the recording or visual types or both as well as to a needle valve and inflation pump. The combination of pressure chamber, needle valve, and inflation pump may com-

municate with or be cut off from the rest of the pneumatic system by the setting of valve "A."

The piezo-electric transducers are essentially differential rochelle salt crystal microphones. A rochelle salt crystal microphone is normally incapable of withstanding any appreciable pressure without probable damage to the crystal. In an application such as this, it is essential to employ some pressure in the registration chamber. As a result, the registration chamber pressure is allowed to communicate with the front and rear of the diaphragms in the piezo-electric transducers when valve "B" is in the "open" position. When the front and rear pressures are identical, the diaphragms are not distended and the crystals in turn are not flexed. Should valve "B" close, the trapped pressure to the rear of the diaphragms will be maintained identical with the pressure in the registration chamber. Any arterial stimulations that are now applied to the registration chamber of the cuff will distend the diaphragms and flex the crystals in proportion to the differential pressure.

A detailed technical discussion of the design and mathematical theory of the differential piezo-electric crystal transducers suitable for the registration of the Korotkow sounds and the arterial pulsations in sphygmomanometry is obviously beyond the scope of this paper. However, a general consideration of some of the more important features should be of interest.

The heart of the differential piezo-electric transducer is a cartridge or diaphragm type of crystal unit similar to that described by Rappaport and Sprague.<sup>56</sup> "It consists of a diaphragm which is directly coupled to a bimorph rochelle salt crystal through a tiny connecting rod; the crystal and connecting rod are located in the interior of the cartridge. Should an actuating force be applied to the diaphragm, proportional stresses will be set up in the crystal, and it will transform these stresses into equivalent electrical potentials which are conducted away from the crystal by means of metallic foil electrodes in contact with it."

According to Rappaport and Sprague, "the fundamental frequency, or natural period, of the bimorph crystal is approximately 10,000 cycles per second. When a diaphragm is coupled to the crystal, the fundamental frequency of the combination is lowered to a few thousand cycles per second." This is well above the upper frequency limits which are present in the Korotkow sounds and in the arterial pulsations.

The attenuation effects throughout the registration frequency spectrum are governed by the electrical, electronic, and galvanometric systems. The channel which registers the Korotkow sounds is identical with that described by Rappaport and Sprague wherein the over-all frequency response of the system is similar to that of the "average acoustic stethoscope." The over-all frequency response of the arterial pulsation registering channel is identical to the sphygmographic arrangement described by Rappaport and Sprague which is flat from practically zero cycles per second to the upper limits of the galvanometric system. An electrocardiographic channel (electronic type) is used for the registration of the arterial pulsations. An electronic type electrocardiographic channel normally possesses a speed of response of 0.01 second or faster.



The method of Miller and White<sup>57</sup> is employed for making possible the utilization of a piezo-electric crystal for the registration of frequency components which approach zero cycles per second, such as are encountered in arterial pulsations.

The essential difference between the piezo-electric transducers employed in this sphygmomanometric apparatus and the sphygmographic systems described by Miller and White and Rappaport and Sprague is the differential arrangement which permits operation at pressures above or below atmospheric.

In Figure 1B is shown the sphygmomanometric apparatus used in this investigation connected to an electronic type electrocardiograph—phonocardiograph.

#### OPERATING TECHNIQUE

The double chamber cuff is applied to the subject as is done with the usual type of sphygmomanometer (Fig. 1A). With tandem valves "A" and "B" open, and the needle valve closed, the system is inflated by means of the pump. When the valves are in this position, all the component pneumatic channels are in direct communication and under identical pressure; the pressure is indicated

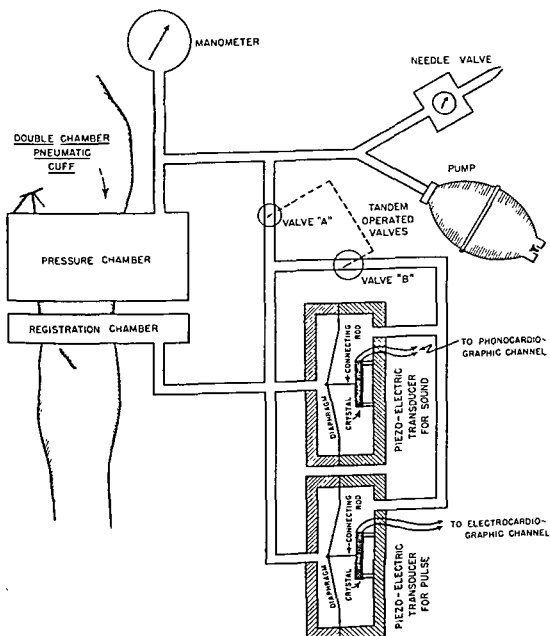


Fig. 1A.—Schematic diagram of an apparatus which is capable of registering simultaneously all of the physiologic phenomena that must be evaluated when estimating blood pressure by the auscultatory, palpatory, and oscillatory methods.

by the manometer. When a pressure of about 20 mm. of Hg is attained, tandem valves "A" and "B" are closed. The closure of valve "A" segregates the pressure chamber of the cuff, the manometer, the needle valve, and pump from the registration chamber of the cuff, valve "B," and the piezo-electric transducers. The closure of valve "B" traps a noncommunicable pressure or cushion of air at the rear of the diaphragms in the piezo-electric transducers equal to the pressure in the registration cuff and the front of the diaphragms.

The pressure is now increased in the pressure chamber of the cuff by means of the pump to above the anticipated systolic pressure. By means of the needle valve, the pressure chamber is gradually deflated. During the deflation period, the registration chamber of the cuff detects the Korotkow sounds and pulse when present and transmits both to the piezo-electric transducers.

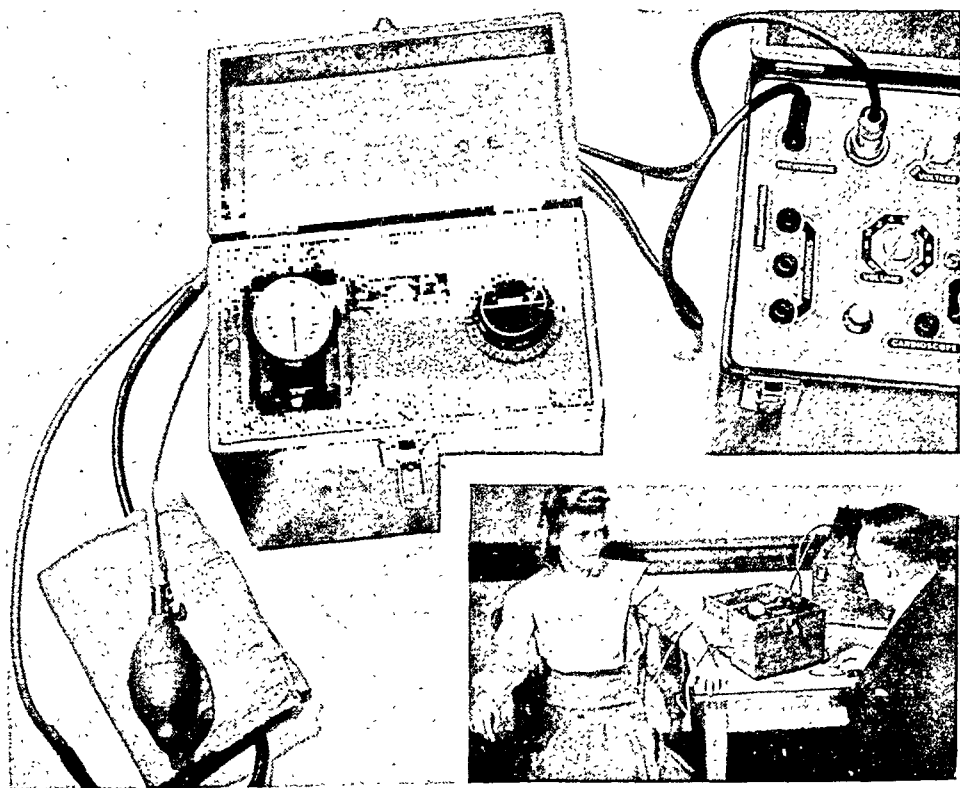


Fig. 1B.—Sphygmomanometric apparatus used in this investigation connected to an electronic type electrocardiograph-phonocardiograph.

The piezo-electric transducer for sound and its associated phonocardiographic system is sensitive to the vibratory frequencies present in the Korotkow sounds but not the low-period vibrations present in the pulse. On the other hand, the piezo-electric transducer for pulse and its associated electrocardiographic system is sensitive to the low-period vibrations present in the pulse. The result is a simultaneous registration of the Korotkow sounds, the pulse, and the cuff pressure (pressure chamber) during the deflation period. It should be mentioned at this point that the phonocardiographic registration system records

the Korotkow sounds with the same degree of modification<sup>56</sup> as produced by the average acoustic stethoscope; logarithmic registration<sup>62, 63</sup> may be used as well.\*

#### THEORY AND OBSERVATIONS

Wiggers<sup>64</sup> in his description of the central arterial pulse and its conversion into the peripheral arterial pulse states: "The central pulse, as the pulse in the large arteries near the heart is called, shows (Fig. 2) first two preliminary vibrations: One due to auricular systole, *a-b*, the other to the isometric rise of tension in the ventricle, *b-c*. These are followed by a sharp primary oscillation, *c, d, e*, 0.013 to 0.02 second in duration and beginning at the ejection of blood

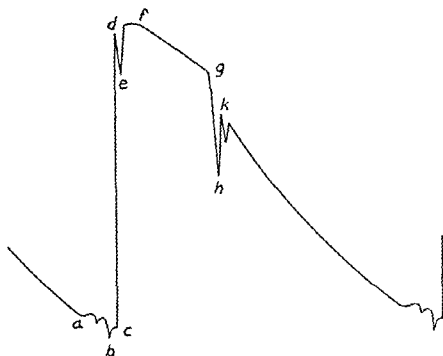


Fig. 2.—The central arterial pulse (after Wiggers).

*a, b*, Vibrations due to auricular systole.

*b, c*, Vibration due to the isometric rise of tension in ventricle.

*c, d, e*, Oscillation due to sudden ejection of blood

*e, f*, Pressure rise when ventricles and arteries form a common cavity.

*f, g*, Gradual pressure fall during rest of systole.

*g, h*, Sudden drop of pressure at the beginning of the ventricular relaxation (diastole)

*k*, After vibrations of valves and blood column.

into the aorta. It is due to the fact that the sudden ejection has set the arterial column in vibration. After this vibration the arterial curve follows the intra-ventricular pressure, for now ventricles and arteries are a common cavity. First, the pressure rises, *c, f*, thereafter reaches a more or less definite summit, and then falls gradually during the rest of systole, *f, g*. At the beginning of the ventricular relaxation (diastole), there is a rapid backward movement of the blood toward the heart, causing the pressure to fall suddenly, thus creating the incisura of the central pulse, *g, h*. Several after-vibrations of the valves and blood column, *k*, recognized over the chest as heart sounds, follow. The pressure then falls smoothly except for a few oscillations due, no doubt, to reflections from the peripheral bifurcations of arteries [Frank<sup>65</sup>]."

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phonocardiographic registration does not register the sound free, but as they are presented to the ears of an observer. Logarithmic phonocardiography is a graphic representation perceived by the average observer of normal hearing when is employed. Logarithmic phonocardiography is thus the human hearing modifications.

"These complicated series of pressure variations present in the aorta and large arteries are modified in their peripheral transmission by friction and interference with reflected waves (Frank). Or, one may state the case differently by saying that the vascular system represents a manometer system, the ability of which to transmit the pressure variations in the central arteries faithfully to the periphery becomes less and less as the length of the column increases [Weber<sup>66</sup>]."

"The changes actually noted as we pass, step by step, to the peripheral vessels are: The preliminary and primary oscillations are damped and obliterated; the sharpness of the incisura is reduced and finally replaced by a rounded dicrotic dip and elevation; the upstroke is delayed and becomes more gradual; the tops are more rounded and the amplitude smaller until in the smallest arteries and capillaries the pulse is entirely obliterated."

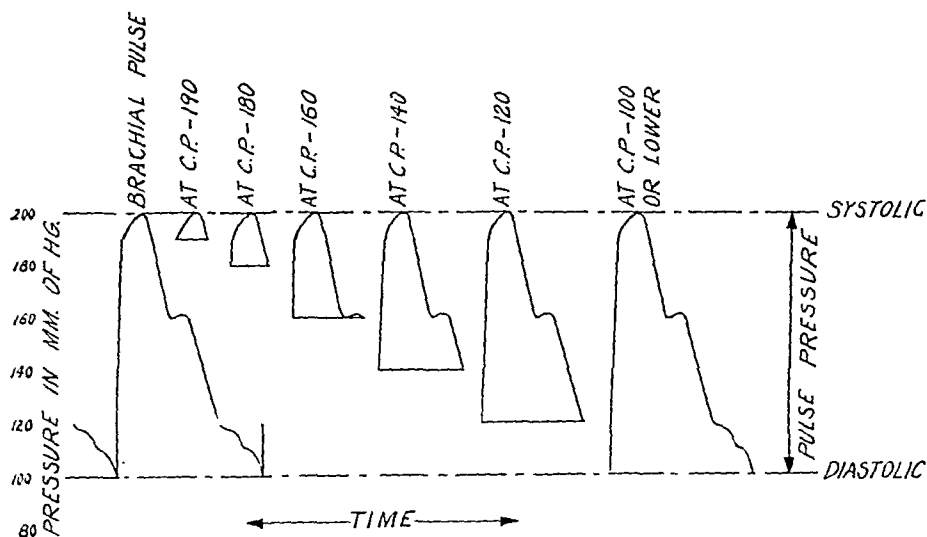


Fig. 3.—A graphic representation of the degree of modification various cuff pressures (above diastolic) produce upon the brachial pulse.

Wiggers further states: "Frank, however, finds difficulty in interpreting the dicrotic wave as merely the incisura and after-vibration modified in transmission, for the dicrotic wave becomes larger toward the periphery and all other waves submitted to the same frictional influence become smaller. He, therefore, believes that its amplitude is augmented by a resonance effect with other vibrations reflected from the periphery."

When a pneumatic blood pressure cuff is applied over the brachial artery, inflated to a pressure above systolic, and allowed to deflate gradually, as is done when estimating the blood pressure, the pulsation in the brachial artery immediately below the cuff is modified. Fig. 3 is a graphic representation of the degree of modification at various cuff pressures. The pulsation marked "brachial pulse" is the usual configuration of the pulse in the brachial artery in which many of the finer oscillations so evident in the central pulse are completely attenuated.

Fig. 3 indicates graphically that when the cuff pressure is above the systolic level, 200 mm. of Hg in this case, the brachial artery is collapsed and no pulsa-

tion is detectable immediately below the cuff. With a cuff pressure slightly less than the systolic level (190 mm. of Hg), a slight pulsation is present in the brachial artery just below the cuff. This pulsation represents the uppermost portion of the brachial pulse, the pressure level of which exceeds the cuff pressure and allows the collapsed brachial artery to open slightly during this short interval. As the cuff pressure is lowered, more of the brachial pulse is passed through the artery because the artery below the cuff is in a collapsed condition a shorter period of time. When the cuff pressure approaches the diastolic level or below, only then is the brachial pulse completely transmitted and at no time during the cardiac cycle is the brachial artery collapsed.

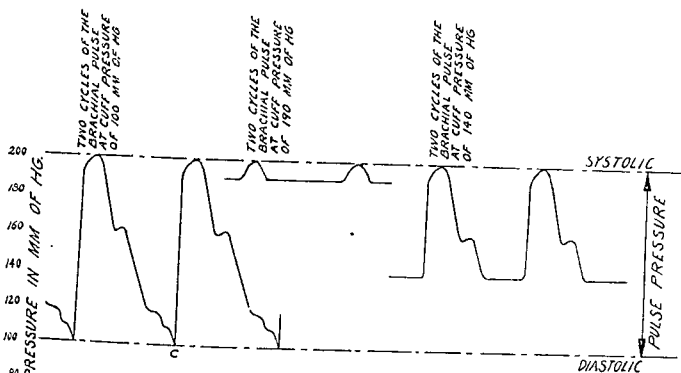


Fig. 4.—Theoretical configuration of two succeeding cycles of the brachial pulse at different values of cuff pressure (above diastolic).

Thus, the systolic pressure in the brachial artery may be estimated by the introduction of a sphygmographic device which is capable of detecting the pulsation of the artery just below the pressure cuff. That is, the systolic level is the point at which the first trace of brachial pulsation is detectable as the cuff pressure is gradually lowered from above the systolic level. This corresponds to the pressure level where the uppermost portion or crest of the brachial pulse (Fig. 3) is greater than the cuff pressure and the artery opens slightly during that instant. In Fig. 4 are shown two succeeding cycles of the brachial pulse (blood pressure 200/100), two cycles at a cuff pressure of 190 mm. of Hg, and two cycles at 140 mm. of Hg. Note the theoretical configuration of the sphygmogram when the systolic pressure is more than the cuff pressure. Also note the manner in which the lower pressure components of the pulse are obliterated by a flattening process due to the collapse of the artery when the cuff pressure is more than the diastolic level. This sphygmographic characteristic permits the estimation of the diastolic level<sup>11</sup> in the following manner: As the cuff pressure is gradually dropping from above the systolic level, the recorded brachial artery pulsations become larger and larger, but a rather flattened base line is present between adjacent cycles until the diastolic level

is reached where the flattening effect due to artery collapse vanishes. The cuff pressure at which the first brachial pulse appears with a well-defined and peaked contour (as represented by *c* in Fig. 4) is the diastolic level.

Let us now analyze an actual brachial pulse tracing as the cuff pressure is gradually lowered from above the systolic level to well below the diastolic level (Fig. 5). The graphic record was obtained with the apparatus shown in Figs. 1A and 1B. The registration cuff was inflated to a pressure of 20 mm. of Hg, and the pressure cuff was varied as indicated on the record.

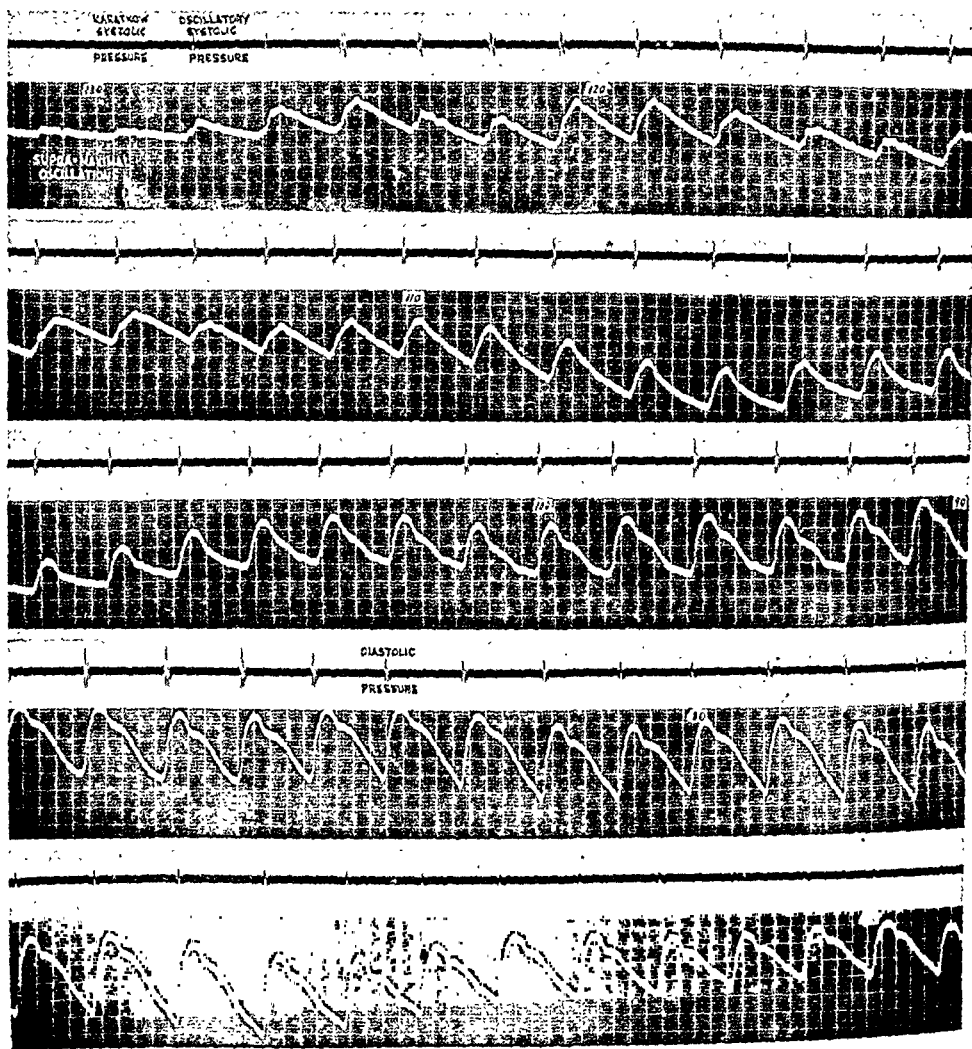


Fig. 5.—A simultaneous registration of the Korotkow sounds and the brachial pulsations (oscillatory curve) during a cuff deflation from above systolic blood pressure to well below the diastolic pressure level. The pressure in the pressure cuff is indicated by the figures in the white squares; the registration cuff was kept at a constant pressure at 20 mm. of Hg. Stethoscopic registration was employed in the sound track.

Fig. 5 shows the typical supramaximal oscillations\* of small amplitude which are followed by a series of gradually increasing pulsations. The first

\*The supramaximal oscillations are not due to passage of blood beneath the cuff but are produced by the "ram action" of the preliminary vibrations *a - b* and *b - c* of Fig. 2 against the cuff. Mechanical vibrations which are detected by the registration cuff are thus set up in the arm. The supramaximal oscillations definitely precede the sharp primary oscillation which is clearly shown in Fig. 5.

distinct pulsation after the supramaximal oscillations is obviously the level of systolic pressure. As indicated in Figs. 3 and 4, a gradual increase in the amplitude of the brachial pulse is expected as the cuff pressure is gradually lowered. The pulse amplitude seems to reach a maximum amplitude between approximately 90 and 70 mm. of Hg cuff pressure and thereafter attains a constant amplitude of pulsation. This is exactly what one would expect to see in an oscillographic curve from a theoretical viewpoint, except for the increase in amplitude between approximately 90 and 70 mm. of Hg. As previously mentioned, Marey<sup>27</sup> attributed this phenomenon to a resonance effect where the arterial wall oscillates freely between two equivalent pressures or what he believed was the diastolic level. Such a resonance effect obviously does take place and is clearly shown in practically all the graphic records we have obtained. This resonance effect, however, is too broad to be useful for accurately evaluating the level of the diastolic pressure. The "flattening effect" just above diastolic pressure and the distinct appearance of a "negative dip" (observed by von Recklinghausen<sup>58</sup> and Bazett and Laplace<sup>11</sup>) just below the diastolic level is more accurate. Note how easy it is to locate this point in Fig. 5, wherein it falls roughly halfway in the resonance region. Theoretically, the termination of the flattening effect should occur exactly in the center of the resonance region.

In Fig. 5, a very faint Korotkow sound appears somewhat earlier than does the first distinct brachial pulsation. Thereafter, a Korotkow sound appears with every brachial pulsation. After the faint first Korotkow sound, the Korotkow sounds gradually increase in intensity for a period of time as the cuff pressure is gradually lowered.

The Korotkow sounds occur simultaneously with the sharp primary oscillation of the brachial pulse or during the beginning of the ejection of blood into the artery. During the rapid ejection interval, the blood velocity is greatly increased when passing through the partially collapsed or constricted portion of the artery immediately below the pressure cuff. This rapid blood velocity in turn sets the artery and surrounding tissue into vibration with the resulting sound. Another contributing factor to the production of the Korotkow sound is the rapid distention of the artery and surrounding tissue by the sharp primary pulse oscillation. A rapid displacement of tissue in a relatively confined area bounded by the nondistensible cuff will produce audible sound waves.

As the cuff pressure is gradually lowered from the systolic level, the arterial pulsations and the Korotkow sounds gradually increase in intensity. A broad resonance effect as previously described occurs in the arterial pulsation in the region of the level of diastolic pressure and thereafter maintains a more or less constant amplitude. The Korotkow sounds, however, suddenly become diminished in intensity at the diastolic level and thereafter become less and less intense. This Korotkow sound intensity effect at the diastolic pressure level may be explained by the two primary causes for its production. That is, when the cuff pressure is less than the diastolic level, there is no longer any arterial constriction with abnormally increased blood flow velocity immediately below the cuff. Also, the rapid distention of the artery and surrounding tissue by the sharp primary oscillation takes place in a relatively less and less confined area as the cuff pressure is gradually lowered. In other words, the Korotkow sound

which is produced when the cuff pressure is just above the diastolic level has both sound generating factors operating at maximum efficiency. But, when the cuff pressure is just below the diastolic level, the former becomes minimal and the latter gradually reduces with the gradual lowering of the cuff pressure.

It is of interest to note that in the blood pressure record of Fig. 5, the first Korotkow sound precedes the first distinct arterial pulsation by approximately 2 mm. of Hg. The abrupt dropoff in the intensity of the Korotkow sound which indicates the diastolic blood pressure level corresponds exactly with the termination of the negative flattening effect of the brachial pulse. In some persons, the first distinct Korotkow sound may precede the first distinct arterial pulsation by as much as 5 mm. of Hg, and vice versa; the discrepancy at diastolic level rarely exceeds 5 mm. of Hg.

Another sound that is commonly registered in the blood pressure graph occurs simultaneously with the incisura of the arterial pulse, the notch preceding the dicrotic wave. The mechanism of the production of this sound is identical to that of the Korotkow sound. Its intensity is much less than the Korotkow sound because the energy generated by the sudden drop in pressure is obviously of lesser magnitude than that due to the rapid ejection of blood into the aorta. The secondary sound which occurs simultaneously with the incisura may be seen in most of the cycles of Fig. 5 but is most distinct below the diastolic level.

Considerable error may be introduced when estimating the systolic blood pressure by the palpatory method. The degree of error may be in the order of several millimeters of mercury below the actual value because the primary oscillation which is palpated is so slight in intensity that it falls below the threshold of human feeling. The diastolic level definitely cannot be determined by palpation, because the sense of human feeling cannot differentiate the point of transition where the negative flattening effect becomes peaked. To depend upon the resonance effect for diastolic blood pressure evaluation is obviously uncertain by the palpatory method because the sense of human feeling is insufficiently sensitive for the accurate detection of the apex of resonance; even graphic registration cannot be depended upon because of the broad resonance effect. Furthermore, it is doubtful whether this resonance effect is palpable in most human beings.

The oscillatory method is reliable in the estimation of the systolic blood pressure only when accurate sphygmographic registrations are made, wherein the phase corresponding to the ejection of blood into the aorta is easily differentiated from the supramaximal oscillations. The sharp primary oscillation, when it first appears at systolic level, is very difficult to differentiate from the supramaximal oscillation by means of a visual type indicator. Usually, the first noticeable sharp primary oscillation via a visual indicator is several mm. of Hg below the actual systolic level. The graphic method requires a paper or film speed of not less than approximately 25 millimeters per second. Slower speeds bunch the component pulse waves too close together making it rather difficult to determine the initial occurrence of the sharp primary oscillation. In most persons the diastolic pressure may be estimated by the negative flattening effect; this phenomenon is not distinct in a small percentage of human beings when using the graphic method and rarely observable via the visual method.



Most all recorded oscillometric curves that are described in the literature show a gradual tapering off of the pulse amplitude as the cuff pressure is lowered gradually below the diastolic level. Our registrations do not show this tapering-off characteristic. *The reason the pulse tapering-off phase is registered by most oscillometers is not because the pulse vanishes, but because the efficiency of the cuff in detecting the arterial pulsations diminishes as the cuff pressure is lowered.* Therefore, it is incorrect to use the pressure cuff as a detector of arterial pulsations as well as a means for collapsing the artery. The use of independent cuffs, one as a pressure cuff for collapsing the artery and the other for detecting the pulsations, is the more accurate method. The latter cuff, however, must be kept at a pressure well below the diastolic level. We have found a pressure of approximately 20 mm. of Hg generally satisfactory. Other well-known sphygmographic techniques may be employed for registering the arterial pulsations, but they are not so simple and convenient to use as is the separate registration cuff method.

When estimating the blood pressure by the auscultatory method, several conditions exist which may introduce error. As a general rule, the systolic pressure is underestimated, because the first Korotkow sound which appears when the cuff pressure is gradually lowered from above the systolic level possesses an intensity below the threshold of human audibility. The auscultatory method is based upon the supposition that the systolic blood pressure level is determined when the artery opens momentarily at the peak of the systolic wave and allows a small quantity of blood to pass below the pressure cuff. The mechanical and hemodynamic factors responsible for the Korotkow sound at this instant are of low magnitude. Thus, the sound level of the first Korotkow sound must be correspondingly low in intensity, and it so happens that in a majority of human beings this intensity is below the threshold of human hearing. Under such conditions, the first audible sound cannot always be considered as representing the level of systolic blood pressure. Yet, this is common clinical procedure. Furthermore, the threshold of human hearing is variable and is dependent upon such factors as:

1. The efficiency of the observers hearing.
2. The efficiency of the stethoscope.
3. The type of stethoscopic chest piece employed.
4. The application pressure of the stethoscopic chest piece.
5. The general noise level in the room.
6. Auscultatory experience
7. The mental and physical state of the observer, especially with respect to fatigue at the time the reading is taken.

We have observed, that as a general rule, the first audible Korotkow sound (heard by a competent observer under optimum conditions) is approximately 5 mm. of Hg below the first actual Korotkow sound that appears; rarely is the differential more than 10 mm. of Hg. Under less favorable conditions, the differential may be considerably greater.

The diastolic pressure as judged by the Korotkow method (when the sounds suddenly become dull and muffled) corresponds exactly with the recorded values and within close limits as compared with the negative transition effect of the arterial pulsation. *Any other sound phase does not bear any relationship whatsoever with diastolic pressure.*

An interesting observation in the registration of the Korotkow sounds is that the five phases are registered by both stethoscopic<sup>56</sup> and logarithmic<sup>62, 63</sup> phonocardiography. However, the stethoscopic method indicates the first Korotkow sound (systolic level) most distinctly, whereas the logarithmic method shows the sudden dull and muffled phase (diastolic level) more distinctly. The murmur components of the Korotkow sounds are best registered by the logarithmic method.

The effects that have been observed by von Recklinghausen, Gumprecht, Müller and Blauel, Janeway, and Ragan and Bordley with respect to the width of the pressure cuff correspond with our observations.

#### SUMMARY AND CONCLUSIONS

1. An apparatus was devised which can register graphically and simultaneously all of the physiologic phenomena which must be evaluated for the estimation of blood pressure by the palpatory, oscillatory, and auscultatory methods. This graphic device eliminates the human element with its subjective differences in the appreciation of the physiologic signs and makes possible a quantitative comparison of the associated phenomena.

2. The sphygmomanometric device used in this investigation is described.

3. The theoretical principles fundamental to the palpatory, oscillatory, and the auscultatory methods are discussed.

4. Following is our summary and conclusions on the *oscillatory blood pressure method*:

A. In an oscillometric curve, the configuration of the pulse wave is modified between the limits of systolic and diastolic pressure. The degree of modification is a function of the systolic, diastolic, and cuff pressures. The wave form is unmodified only when the cuff pressure is lower than the diastolic pressure level.

B. Diastolic pressure is represented in the oscillometric curve by the first arterial pulsation which is undistorted during its most negative phase.

C. The negative transition effect is rarely observable via the visual oscillometric method.

D. The resonance effect which occurs in most oscillometric registrations corresponds to the level of diastolic pressure. Although this resonance effect is observable in most persons, it is much too broad to be useful as an accurate indication of diastolic pressure.

E. The oscillatory method is reliable in the estimation of the systolic blood pressure only when accurate sphygmographic registrations are made. Inaccurate or poor sphygmograms do not indicate distinctly the initial appearance of the minute wave which represents the beginning of ejection

of blood into the artery as differentiated from the supramaximal oscillations.

F. The gradual tapering-off of the pulse amplitude in an oscillometric curve below the diastolic pressure level commonly described and illustrated in the literature is not a physiologic phenomenon but a definite *instrumental error*. The arterial pulse does not gradually diminish and then disappear below the diastolic pressure level, but remains more or less of constant amplitude. The instrumental error is essentially due to a *diminution in the efficiency of the cuff as a detector of arterial pulsations* as the pressure is lowered. The use of independent pressure and registration cuffs eliminates this source of error.

5. Following is our summary and conclusions on the *Korotkow method*:

A. The Korotkow sounds occur simultaneously with the sharp primary oscillation of the brachial pulse which is the beginning of the ejection of blood into the artery.

B. There are two major contributing factors to the production of the Korotkow sounds; the mechanism of each is described.

C. The first distinct Korotkow sound (systolic pressure level) generally occurs simultaneously with the first distinct sharp primary oscillation of an oscillometric curve. The first suddenly diminished Korotkow sound (auscultatory diastolic pressure level) corresponds very closely to the first undistorted arterial pulsation.

D. An explanation is given for the sudden diminution in the intensity of the Korotkow sounds at diastolic pressure.

E. A secondary sound that is commonly registered, although seldom heard, occurs simultaneously with the incisura of the pulse curve. The mechanism of the production of this sound is similar to that of the Korotkow sounds.

F. When estimating blood pressure by the auscultatory method, seven major conditions exist which may alter the threshold of hearing and introduce error.

G. As a general rule, the systolic pressure is underestimated because the first Korotkow sound which appears during a gradual cuff deflation possesses an intensity below human audibility.

H. Diastolic blood pressure as judged by the auscultatory method (when the sounds suddenly become dull and muffled) corresponds exactly with the recorded values and within close limits with the negative transition effect of the oscillometric curve. *Any other sound phase does not bear any relationship whatsoever with diastolic pressure.*

I. The stethoscopic method of sound registration shows the first Korotkow sound (systolic pressure level) most distinctly, whereas the logarithmic method registers the sudden diminution (diastolic pressure level) more distinctly than the stethoscopic method.

J. The murmur components of the Korotkow sounds are best registered by the logarithmic method.

6. The effects that have been observed by von Recklinghausen, Gumprecht, Müller and Blauel, Janeway, and Ragan and Bordly with relation to the width of the pressure cuff correspond with our graphic data.

7. In some instances, the sudden muffling effect at the diastolic pressure level is not audible. In such cases, the graphic method may show this diminution effect with sufficient clarity to accurately estimate the diastolic blood pressure. We have never encountered a condition where the combination of auscultation, graphic registration of the Korotkow sounds, and the graphic oscillatory method did not distinctly indicate the diastolic pressure level; that is, at least one of the methods always gives a distinct and clear indication.

8. Considerable error may be introduced when estimating the systolic blood pressure by the palpatory method. The degree of error may be in the order of several millimeters of mercury below the actual value, because the initial primary oscillation is below the threshold of human feeling.

9. The diastolic pressure level cannot be estimated by the palpatory method, because the sense of human feeling cannot differentiate the point of transition where the negative flattening effect becomes peaked. The negative transition effect is the only reliable oscillometric method for the estimation of diastolic blood pressure.

10. The graphic registration method we have described, which registers all of the physiologic phenomena associated with indirect sphygmomanometry, has application in the more exact studies of blood pressure. The method shall prove useful in the clarification of such phenomena as the auscultatory gap, the double tone of Traube, the double murmur of Duroziez, the murmur accompanying the pistol shot pulse, etc.

We wish to express our appreciation for the cooperation of the Sanborn Company, of Cambridge, Massachusetts, in this investigation.

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### Erratum

In the article by Rappaport and Rappaport entitled "An Improvement of the Sero-logic Kahn Reaction in the Spinal Fluid" in the August, 1943, issue of the JOURNAL, the procedure (second line from the bottom of page 1355) should read as follows:

Pour 0.1 c.c. of serum (reagent II) in each of two small test tubes and inactivate it at 56° C. for twenty minutes, simultaneously with a third tube containing a little more than 1.5 c.c. of spinal fluid.

## APPARATUS FOR THE FREEZING-DRYING OF TISSUES FOR STORAGE\*

A. CECIL TAYLOR, PH.D., CHICAGO

**F**REEZING-DRYING has been employed successfully as a method for preserving segments of nerve and artery to be used later as grafts in experimental animals.<sup>1, 2</sup>

In order to reduce to a minimum distortion of the cellular structure of tissues subjected to freezing and drying, as well as chemical changes during the subsequent period of storage, three conditions are of prime importance: (a) To freeze the whole tissue rapidly, (b) to dehydrate as completely as possible, and (c) to maintain a low temperature during the dehydration process. Aside from appropriate beakers and thermos flasks, no special equipment is needed for the quick freezing of tissues. However, the subsequent process of dehydration in high vacuum at a constant low temperature requires special apparatus.

Based upon the principles of the equipment used by Bensley and Gersh<sup>3</sup> and Hoerr,<sup>4</sup> a freezing-drying unit was built by the author embodying certain simplifications and alterations of design which adapt it for the particular use intended. Aside from the low cost and simplicity of the apparatus here described, its chief advantage for our use lies in the fact that the tubes in which tissues are desiccated are easily accessible and can be sealed off without breaking the original vacuum. The dried tissues are thus protected from any contact with atmospheric air and moisture.

The apparatus consists of two systems, a vacuum desiccation system and a cooling system.

### THE DESICCATION SYSTEM

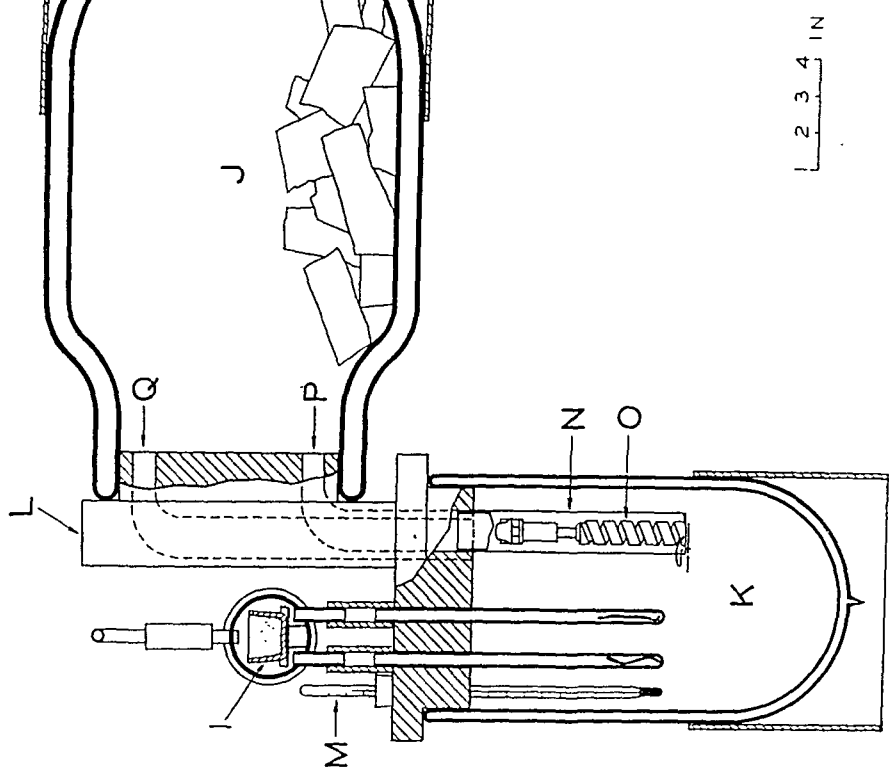
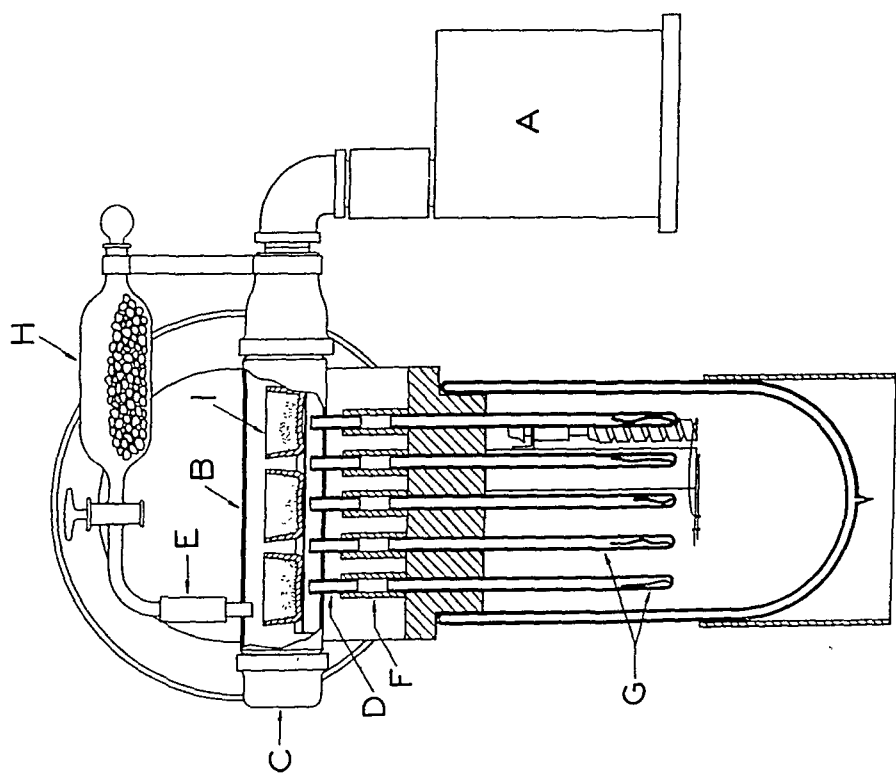
The vacuum for desiccation is maintained by a Cenco high-vac pump. This has proved satisfactory when used alone, although the addition of a mercury diffusion pump in series would shorten the time required for drying the tissues at the low temperature necessary.

The pump (A, Fig. 1) is connected directly with a manifold (B) constructed of 2-inch galvanized iron pipe and fittings. The manifold is furnished with 9 nipples (D) for the attachment of the vials containing the tissues, and one (E) which may serve as an air inlet or as a place for the attachment of a vacuum gauge. All threaded joints were carefully sealed to insure against leakage. Since the movement of gases at very low pressures is greatly retarded by long, small caliber channels, the vacuum system was designed with the largest feasible inside diameters and shortest possible distances with the elimination of "bottle necks."

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1 2 3 4 IN

FIG. 1.



To protect the pump from moisture and to speed desiccation of the tissue, phosphorus pentoxide is placed in the manifold in dishes (*I*) set in a tray which can be removed from the manifold after unscrewing the manifold cap (*C*). Hermetic seal of this end of the manifold is insured by inserting into the screw cap a closely fitting steel disk and a rubber gasket.

Unless a gauge is to be used in the vacuum system, the extra nipple (*E*) is connected with a calcium chloride air dryer (*II*) controlled by glass stopcocks. This serves as an air inlet whenever it becomes necessary to break the vacuum before sealing off the tissue vials, e.g., in recharging the manifold with fresh phosphorus pentoxide during a run.

The frozen tissues are dehydrated in vials 20 cm. long, made from pieces of 10 mm. stock pyrex tubing by sealing one end in a flame. Nine of these tissue vials (*G*) may be attached to the manifold at one time by means of rubber pressure hose connections (*F*).

#### THE COOLING SYSTEM

In the preservation of bacteria suspended in liquid, or of fluids such as plasma, the cooling effect due to the evaporation within the evacuated tubes is sometimes depended on to maintain the material in a frozen state.<sup>5</sup> However, in the case of dense tissues such as nerves, with relatively low water content, evaporation is not sufficiently rapid to keep the temperature securely below the freezing point of the protoplasm and intercellular fluids. Especially is this true as dehydration progresses and the electrolytes of the tissue become more concentrated with a consequent lowering of their freezing point. For such tissues the highest temperature at which dehydration may be carried on without too rapid growth of ice crystals is in the vicinity of  $-40^{\circ}\text{C}.$

In the more elaborate systems (Bensley), cooling is effected by mechanical refrigeration units with coils in heavily insulated chambers. These have the advantage of being reliable and accurately adjustable, but are cumbersome and expensive. In the apparatus here described a device for the use of solid carbon dioxide (dry ice) as a cooling agent was constructed. It consists of two thermos flasks connected by convection vents. One flask serves as source of cold gas which is utilized in the other for the refrigeration of the tissues. The gases in the former are kept near the sublimation temperature of carbon dioxide by packing the flask with dry ice, while the temperature in the latter may be maintained at the desired level by controlling the convection currents between the two flasks. The dry ice chamber (*J*) is a 12 liter Pyrex thermos flask laid on its side. A  $4\frac{1}{2}$  liter Pyrex thermos flask with wide mouth provides the constant temperature chamber (*K*) for refrigerating the tissues. Both flasks are closed by a common cover unit (*L*) which contains the convection vents (*P*, *Q*) as well as perforations for the tissue vials. The cover unit, details of which are shown in Figs. 1 and 2, is made of several thicknesses of  $\frac{1}{2}$  inch celotex stock cut to shape, assembled, and held together with large wood screws. This laminated construction provides a simple method of fashioning the gas vents. Several coats of shellac over the outside of the cover unit prevent the seepage of moisture into the system through the pores of the celotex.

The cold gas vent (*P*) is extended downward into the refrigeration chamber (*K*) by a metal tube (*N*) attached to the cover unit. This bears at its lower end

a thin metal damper valve (*S*, Fig. 2) which is adjusted to swing freely on its pivot so as to close off the vent. The damper valve is activated by a bimetallic thermostat (*O*) to which it is connected by a lever (*R*). By turning a knob (*T*) the thermostat may be adjusted to close the valve at any chosen temperature. Care was taken to provide a large cross-sectional area (not less than 5 sq. cm.) for the vents throughout their lengths in order to facilitate rapid convection currents. To permit the insertion of the tissue vials into the refrigeration chamber (*K*), holes were drilled into its cover, an extra hole being provided for the thermometer (*M*).

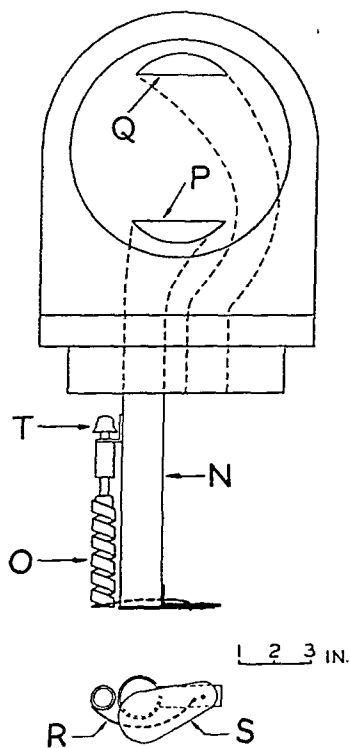


Fig. 2.

In operation, the cold gas mixture (air and carbon dioxide) within chamber *J* passes through the cold gas vent (*P*) and past the open damper valve (*S*) into chamber *K* where it displaces the lighter, warmer gas it encounters there. This rises to the top of the chamber and is forced through the warm gas vent (*Q*) back into chamber *J* where it becomes cooled by contact with the dry ice. As the gas in chamber *K* is cooled, the bimetallic helix is distorted so as to gradually close the damper valve and retard the convection current until an equilibrium is established at the temperature for which the regulator has been set.

A simple wooden framework provides support for both the desiccation and cooling systems (Fig. 3). The pump with its motor and the manifold are rigidly secured to the frame, while the thermos flasks and cover unit are demountable. In assembling these latter the cover unit is raised into the position indicated in the photograph after inserting the tissue vials into the holes provided for them.

The refrigeration flask (*K*) is then brought up over the tissue vials and fitted to its cover. It is supported in this position by drawing under it a sliding shelf. The dry ice flask (*J*) is cradled between the two horizontal members of the frame so that it may be slid onto its cover or pulled back at will.

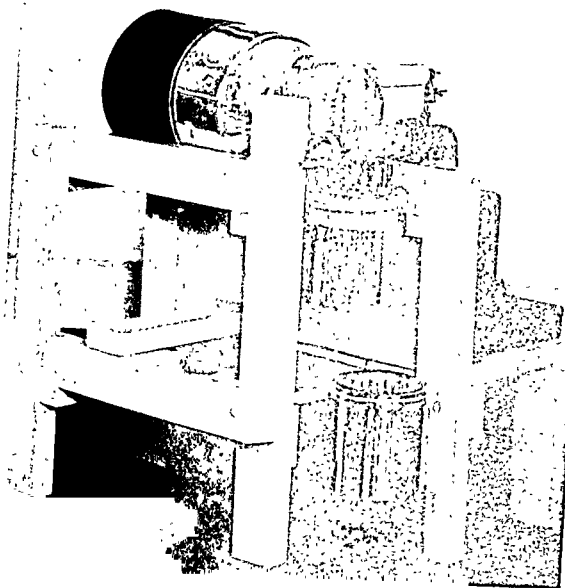


FIG. 3

## OPERATION

Tissues, having been prepared aseptically, are dropped directly into isopentane (practical grade, Eastman Kodak Co.) previously chilled in liquid nitrogen to a temperature of  $-150^{\circ}\text{C}$ . About ten seconds is allowed for complete freezing of the tissue. Since it is important that the temperature of the frozen tissue be kept below  $-40^{\circ}\text{C}$ ., until it has become completely dry, precautions against warming must be observed at each step in the process of transferring the tissue from the isopentane into the assembled cooling chamber. Before removing the frozen tissues from the isopentane, the sterile Pyrex vials are cooled with dry ice. With chilled forceps, the frozen tissues are then dropped into the vials which may be loosely plugged with sterile cotton. After all vials have been filled, they are attached to the rubber couplings on the manifold nipples. This process must be done quickly, handling the cold vials with leather gloves cooled by holding a piece of dry ice for a short time. In order to keep already a vials cool while others are still being handled, a container of alcohol char

dry ice is brought up under the vials until they dip into the liquid. When all vials are attached, the cover unit is raised into position over them, and the refrigeration flask (*K*), which has previously been thoroughly cooled with dry ice, is brought up onto its cover. Flask *J*, filled with dry ice, is now slid into position on its cover. The convection currents begin immediately. The desiccant dishes (*I*) are now filled with phosphorus pentoxide and placed in the manifold whose cap (*C*) is then screwed tight. The vacuum pump is started and evacuation continued until the desiccation of the tissue is completed.

The dry ice flask (*J*) holds between 15 and 20 pounds of solid carbon dioxide when filled to capacity. This amount maintains a temperature of  $-40^{\circ}\text{C.}$ , in the refrigeration chamber (*K*) for about 72 hours. During a long run 10 pounds of dry ice is added every second day by simply sliding flask *J* back from its cover, refilling with dry ice, and returning it to its cover again. One must guard against clogging the cold gas vent in the cover with pieces of dry ice during this refilling process.

The amount of phosphorus pentoxide necessary for a given run depends on the amount of moisture in the tissue to be desiccated. When all vials are heavily charged with moist tissue it has sometimes been necessary to replace the phosphorus pentoxide with fresh powder. To do this the vacuum must first be broken by opening the cocks in the air inlet desiccator (*H*) so as to admit air into the manifold very gradually. After air ceases to enter through the cocks, the cap *C* may be unscrewed and the desiccant dishes removed for refilling.

Most of the moisture extractable by the dehydrator is removed from the tissues during the first 48 hours; however, tissues to be stored for long periods have routinely been allowed to run from two to four days longer. The larger the pieces of tissue, the longer is the time required for drying. Tests with anhydrous copper sulfate show that nerves dehydrated for eight days still contain traces of water. However, it has been shown that, even after much more rigorous freezing-drying procedure, there still remains as much as 1 per cent moisture in the tissue.<sup>4, 6</sup>

The temperature within the refrigeration chamber (*K*) may be regulated by adjusting the thermostat controlling the damper valve. The degree of temperature constancy depends on the sensitivity of the thermostat and damper valve. Variations of no more than  $\pm 2^{\circ}\text{C.}$  are characteristic of the equipment in use in our laboratory, which is entirely adequate for this purpose. If moisture can enter the cooling system through the pores of the cover unit or through improper fit of the covers, snow may form in the vents, thus cutting down, or shutting off completely, the convection current. Should this occur, the snow may be jarred loose by tapping on the metal tube (*N*) or by inserting a rod into the vent from below after removing flask *K*. When the dry ice in chamber *J* is reduced to less than 5 pounds, the temperature of the system begins to rise.

It should be noted that there is a temperature gradient from the bottom to the top of the refrigeration chamber (*K*). Therefore, to determine the temperature of the tissues in the vials, the bulb of the thermometer (*M*) should be on a level with the bottom of the tissue vials.

On completion of dehydration, the vials containing the tissue may be sealed off, either after filling with air through the desiccator (*H*), or while still evacuated. The cooling chambers and cover unit must first be removed. Then with

one vial at a time insulated from the rest by an asbestos sheet, the flame of an oxygen hand torch is played upon the walls of the vial at a point somewhat above the middle. When the glass is softened, the lower part which contains the tissue is drawn away and sealed. If the tissue is to be sealed in vacuum, the pump is left running while each vial, in turn, is shut off from the vacuum manifold by a screw clamp on the rubber connection at *F*. This is done as a precaution against the entrance of moist air into the whole system in the case of accidental perforation of the wall of the vial. Care must be exercised while sealing not to overheat the glass at any point, since, with tubing of this diameter, atmospheric pressure may easily force an opening through the softened glass.

Tissues sufficiently dehydrated and thus sealed in vacuo may then be stored at room temperature.

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Into the bottom of the barrel (*A*) of an ordinary 2 c.c. syringe a soft spring (*B*) is inserted to hold up the plunger (*C*), which has been transformed into an open tube by removing the upper and lower ends. At the upper end of this tube a vaccine-bottle rubber stopper, from which the center has been cut out, is slipped on to serve as container (*D*) for firmly packed cotton and gauze (*E*) which serves as applicator pad. The barrel (*A*) of the syringe is held firmly in the screw top (*F*) by means of the rubber band (*G*). To serve as ball valve, an ordinary steel pin (*H*) with round head is dropped into the bottom of the barrel. The amount of liquid forced up into the container (*D*) at each stroke of the pump can be adjusted by the interposition of rubber washers (*J*) between the bottom of the container and the top of the barrel.

Before starting to inject a series of mice, the Pad (*E*) is pressed down repeatedly until it is soaked with disinfectant. The operator then takes the filled syringe into one hand, picks up a mouse with the other, presses the animal's abdomen gently against the pad (a pressure of ca. 20 Gm. is sufficient), which in turn is pushed downward, and pumps liquid up. The brief gentle touch produces an even round moist spot of disinfectant on the skin of the animal through which the injection is made. The operator then relinquishes that animal, picks up another one, and proceeds similarly without depositing the syringe before it is empty.

*Summary.*—A simple mechanism is described which permits the rapid automatic application of a graded quantity of liquid disinfectant to the abdomen of mice injected intraperitoneally in large series, and which results in considerable saving of time.

## CHEMICAL

### NOTE ON THE SELENIUM METHOD FOR THE DETERMINATION OF NONPROTEIN NITROGEN\*

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THE principal advantage of the method of Reis and Powers<sup>1</sup> for the determination of nonprotein nitrogen in blood is that the digestion tubes do not become etched. The necessity for conserving apparatus makes this feature of value at the present time. Since the publication of the method, several points that need clarification have come to our attention and are set forth in this note.

1. While the selenium-sulfuric digestion mixture prepared from selenious acid gives no trouble, the one prepared by dissolving selenium in sulfuric acid and diluting with water often develops a red amorphous precipitate of selenium. This is caused by insufficient cooling during the addition of the water. While this precipitate does not render the reagent unusable, we now suggest the following method of preparation in which there is little danger that a precipitate will form.

Dissolve with heat 70 mg. of selenium in 10 c.c. of concentrated sulfuric acid. As the selenium dissolves the solution will become first green and then clear with a slight yellowish cast. Cool thoroughly and add 200 c.c. of cold 40 per cent sulfuric acid.

2. The instructions read to have the flame of the microburner 1 inch high and the bottom of the Pyrex tube  $\frac{3}{4}$  inch above the top of the burner. This is commonly interpreted to mean  $\frac{3}{4}$  inch above the top of the flame, whereas it should actually be  $\frac{1}{4}$  inch in the flame. With this adjustment the boiling will be vigorous and continuous, and bumping will not be as likely to occur.

3. In the Folin-Wu method it is common practice to disregard very slight turbidities, but in this method a turbidity that is scarcely visible to the naked eye may introduce a considerable error. However, no turbidity will occur if the tube is thoroughly cooled after digestion, and all subsequent steps in the nesslerization are completed as rapidly as possible. If properly done, the nesslerized solutions may be allowed to stand several hours before reading, with no development of turbidity.

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\*From the Department of Biological Chemistry, Tufts College Medical School.



# MEDICAL ILLUSTRATION

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## FACIAL AND BODY PROSTHESIS IN RELATION TO WAR WOUNDS

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CAPT. CARL DAME CLARKE, A.U.S.

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EVERY war has produced wounds that require both plastic surgical repairs and prosthetic appliances. The advent of high explosives and inflammable mixtures and the extensive use of the airplane have increased greatly the necessity for such work. Although plastic surgery is a relatively new field in comparison with other branches of medicine, the really worth-while advances in prosthetic appliances have been made only in the last twenty years. For this reason, the average physician or surgeon may not ascribe to them the importance they rightly deserve. Often the surgeon considers only plastic surgery, when he has at his disposal a method of repair that is high in cosmetic, psychologic, and physical value.

The purpose of this paper is to bring facial and body prosthetics to the surgeon's attention so that patients may benefit from the possibilities.

The technique of producing these prosthetic appliances has been described previously in considerable detail by Clarke,<sup>1,2</sup> by Bulbulian,<sup>3, 4</sup> and by Kazanjian, Rowe, and Young.<sup>5</sup> Therefore, this part of the subject will not be considered here.

Facial and body prostheses are divided into two main groups as follows:

1. Those that are purely of a temporary nature, for cosmetic purposes only, provided to be worn until the defects can be corrected permanently by plastic surgery. Typical examples are cases in which an artificial tip of a nose or a section of a cheek are made to replace temporarily the actual parts lost through disease or injury. In such instances plastic surgery will be done later and will obviate the necessity for the prostheses.

2. The second group is comprised of cases beyond the help of plastic surgery. A typical example of this is an artificial hand made to replace a missing natural hand. The artificial hand at present available should not be confused with the prosthesis hitherto employed. Formerly the hand was carved from wood or constructed from metal and then covered with a leather glove. It looked unnatural at all times. Now the prosthetic hand is made of rubber, with the shade and texture corresponding to that of a real hand and containing all the skin detail. In fact, a fingerprint impression or pattern can be made as easily from an artificial rubber hand as from a natural hand. Since the rubber is filled with a plastic material which allows the fingers to be bent into any position, it may be used for writing, eating, or for carrying light objects. It, too, can be covered with a leather glove when desirable; however, this is unnecessary because the rubber hand has the same color and texture as the natural one.

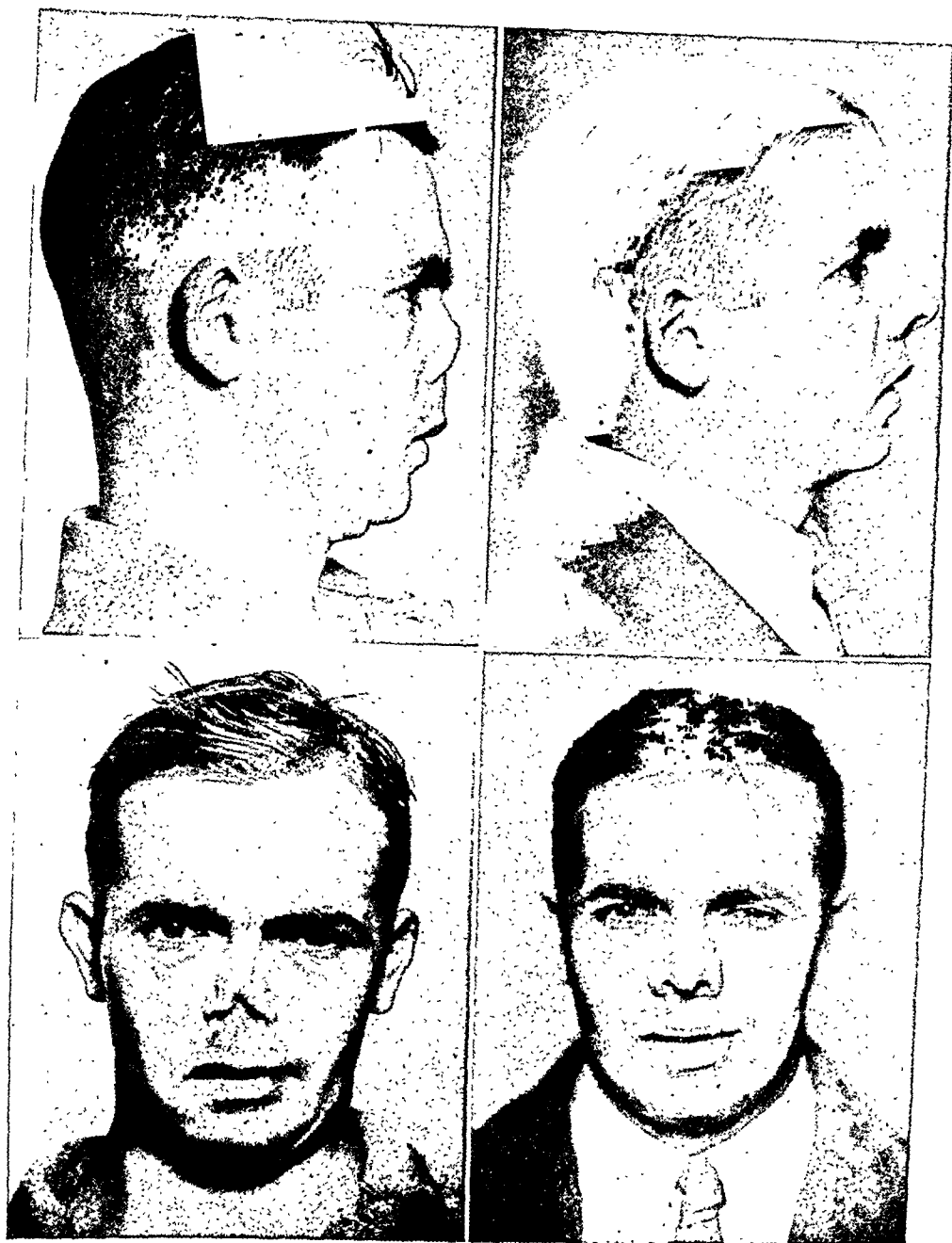


Fig. 1.—Top, profile view of a patient before and during the wearing of a prosthesis of a nose.  
Bottom, a front view of the same patient.

At times the surgeon and prosthetist together should decide which method of repair, surgical or prosthetic, will be most beneficial to the patient. One method alone should not be considered in all cases. In other words, the surgeon and prosthetist should weigh the value, desirability, and debilitating effect to the patient both physically and psychologically. When an entire ear has been destroyed, it may be considered advisable to replace the missing organ with a soft rubber prosthesis, rather than to attempt the reconstruction of the ear by plastic surgery. Such surgical procedure has not, to date, been entirely successful. When it has been attempted, numerous operations have been performed over a period of years. In fact, one exceptionally competent surgeon performed an average of three minor operations a year over a period of fifteen years to reconstruct an ear. It is possible that methods will be perfected in



Fig. 2.



Fig. 3.

Fig. 2.—A profile view of the same patient as seen in Fig. 1. The eyeglasses are used to help hide the join lines where the prosthesis comes into contact with the skin. An adhesive is employed to hold the prosthesis in place.

Fig. 3.—A front view of the same patient, as seen in Figs. 1 and 2, with eyeglasses in place. Dark lenses are unnecessary. In fact, the frames alone are suitable. The prosthesis is not attached in any way to the frames.

the future which will make the prosthetic ear superfluous. The same criticism applies to the reconstruction of the nose when the entire organ has been destroyed. However, if both natural alae remain, a presentable nose may be reconstructed by plastic surgery. Although the surgical procedure may be lengthy and painful to the patient, with considerable detrimental psychological effect, nevertheless the surgical repair becomes a permanent part of the body. It is also logical to reason that should a patient have a minor defect which can easily be corrected by plastic surgery, time and expense should not be consumed in preparing a temporary prosthesis.

In comparison to lengthy surgical procedures a prosthesis requires but a few hours of time to make. It causes the patient no inconvenience or pain during the preparation, application, and wearing. However, it always remains



Fig. 4.—A prosthetic hand. The patient's left hand (the hand on the right in the photographs) is artificial. It is filled with a plastic material which permits the fingers to be bent into desired positions for use.

an artificial part which must be removed, cleaned, and replaced from time to time.

A face prosthesis, if properly compounded and technically executed, will last for years. A prosthetic hand receives more wear and for this reason must be replaced more often. This is of little consequence because at any time, a new hand can be made from the original plaster or metal mold, which is filed away for this purpose. In fact, under favorable conditions, artificial rubber hands can be supplied immediately from stock molds to replace those members

lost by disease or accident. Invariably one of these will be found to suit the patient.

Under no condition should a temporary or permanent prosthesis be made until the normal tissues have healed completely and all evidence of edema and swelling have disappeared; otherwise the final prosthesis will not fit the remaining parts of the body. *Join lines*, or lines formed where the artificial part joins the natural tissues, will become too evident. Cosmetics, make-up putty, and eyeglass frames cannot be relied upon to hide these join lines if the prosthesis does not fit properly. The failure of a prosthesis usually is caused by faulty technique on the part of the prosthetist or by the fact that the mold was made before the swelling and edema subsided. In the latter case the prosthesis will not fit after the tissues have changed in size. When a prosthesis is made to fit over a hairy surface, such as the upper lip or lower jaw, the hair must be kept shaved from the skin where the prosthesis is to adhere to the surface. Otherwise, the hair will force the prosthesis from the surface and result in poor adhesion and visible join lines.



Fig. 5.



Fig. 6.

Fig. 5.—A photograph of a patient without a prosthesis.

Fig. 6.—The same patient as seen in Fig. 5 with a temporary prosthesis in place. A prosthesis of this type is made for psychologic and cosmetic purposes only, until the necessary plastic surgery can be accomplished. In this instance dark glasses were used to hide the eye socket until an artificial eye could be supplied.

When a good adhesive of the proper consistency is used, the patient need have no fear of losing the artificial part; it will cling firmly to the surface. If the prosthesis is removed from time to time and cleaned before it is replaced, there will be little possibility of infection. In fact, no case of irritation or infection from the wearing of a prosthesis has yet been reported. There have been recorded two cases of patients having an idiosyncrasy to the gum mastic used in the adhesive. This difficulty could have been overcome by substituting another gum, such as colophony, which serves equally well. The author has made prostheses for physicians themselves; and, after years of use, they have

reported that no infections or irritations ever developed. One of these physicians removed his prosthesis every night. Another wore his for a period of fourteen days before removing it for cleaning.

The face and hands may be washed while the prosthesis is in place without danger of it coming off or of drastically changing the esthetic effect. However, it is desirable that cosmetics be applied after washing. Finally, it must be realized that a well-compounded prosthesis will not change in color or shade. This does not hold true in regard to skin tones. In summer the skin darkens; in winter it lightens. The patient should therefore be supplied with three prostheses of slightly different colors to allow for the changes of the normal skin. This necessitates but little additional work on the part of the prosthetist. For many rubber casts can be made from the same plaster mold in a short period of time. The application of a lotion or powder base and face powder compensates for the slight color changes that may exist between the actual skin and the prosthesis.

Prosthetic work in the past has been done primarily by dentists, and invariably this has applied to the oral cavity proper. However, some cases, such as wounds of the lower jaw, should have temporary skin prostheses made to prevent the continuous dribbling of saliva until a plastic repair may be accomplished. As soon as the loss of saliva is checked, the patient feels better and gains weight. In turn, this speeds the day on which a plastic procedure may be followed to effect a permanent repair. It must be reasoned that saliva is essential to normal gastronomic functions and its loss impairs the general health of the patient.

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